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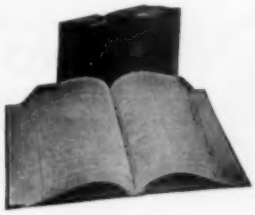

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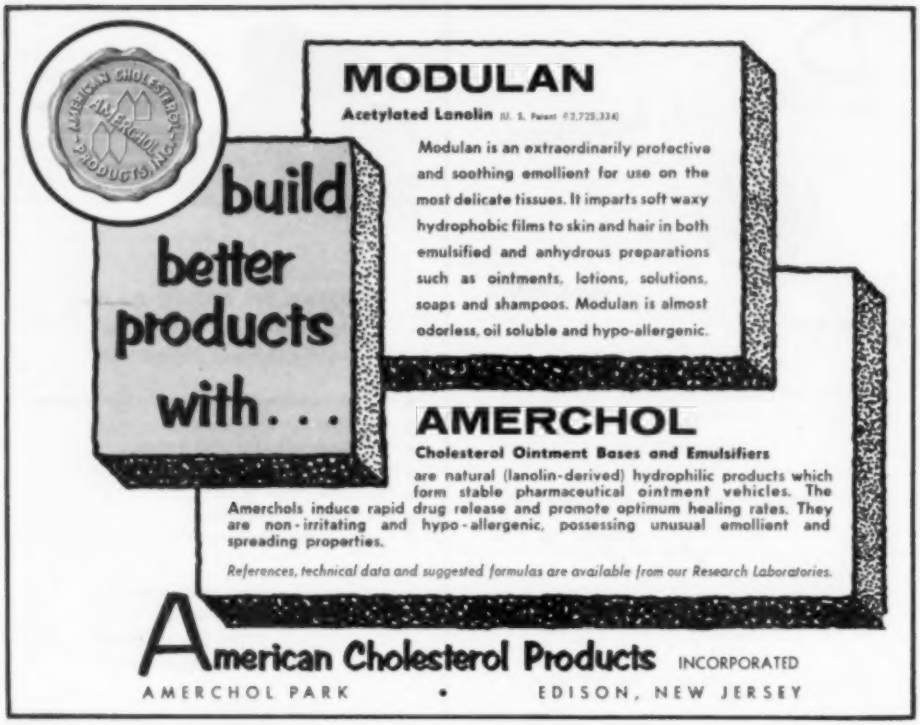
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Drug Addiction II*

Variation of Addiction

By JOHN R. NICHOLS† and W. MARVIN DAVIS

As in previous experiments with this procedure, relapse by animals was demonstrated. Different degrees of drug-directed behavior were produced in groups of rats which underwent escape training. Small injections given between training trials increased the drug-directed behavior, probably because they retarded, but did not prevent, some loss of tolerance and dependence during the procedure. It seems likely that the time interval between training trials is too long and should be shortened for maximal effect on drug-seeking behavior. More important, this experiment indicates that for rats, as for man, the conditions under which morphine is received is more highly related to the development of drug-seeking behavior than is any "inherent rewarding properties" of morphine.

THE DEVELOPMENT of compulsive drug-seeking behavior in man seems related to whether he takes an active role in using the drug. Illegal opiate-users initiate the drug-taking action and this behavior rapidly becomes a chronic obsessive-compulsion. This is not true for the passive recipient of opiates in a hospital setting.

Behavioral techniques which permit animals an active role in opiate use are among the newest methods for studying opiate addiction. Chimpanzees (1) and rats (2) have been shown to develop drug-seeking behavior when suffering withdrawal symptoms. Recently, Nichols, Headlee, and Coppock (3) have shown that escape-training can cause rats to increase their performance of an instrumental act of escape, i.e., drinking a previously rejected morphine solution. Beach (4) has shown that rats develop a preference for stimuli associated with the effects of morphine injection. In both of these experiments, the changes in behavior wrought by the experimental procedure survived complete abstinence from morphine for several weeks.

The explanation of these drug-directed behavioral patterns in the rat has been based upon learning-conditioning principles. Ample evidence, e. g., Skinner (5), Verplanck (6), Thorndike (7), Mowrer (8), and many other conditioning studies (9, 10), supports the following principles: any response which is reinforced tends to be learned and repeated; and the termination of a disagreeable or noxious stimulus (*e. g.*, electric shock, immersion in water, or excessive heat or cold) is a reinforcing circumstance.

If a response is reinforced by the termination of a noxious stimulus, the procedure is called "escape training" and the response is called the "instrumental act of escape" or "escape response." A rat which learns to press a bar to turn off an electric shock is performing an instrumental act of escape which it learns as a result of escape training (9, p. 56).

The disagreeable, distressing, and noxious stimuli of withdrawal symptoms may be terminated by an intake of morphine. Any response, therefore, which results in a morphine-

* Received August 15, 1958, from the University of Oklahoma, College of Pharmacy, Norman.

Based, in part, upon a Ph.D. dissertation submitted to the Graduate College, University of Oklahoma, by the senior author.

† Present address: Southeastern Louisiana College, Hammond.

The authors are indebted to Dr. Irene Mackintosh for many valuable suggestions made during the preparation of this article.

intake will be reinforced by termination of the withdrawal distress and tend to be repeated.

This experiment attempts to manipulate the withdrawal intensity in groups of rats during the course of escape training. Differing degrees of withdrawal intensity should be reflected in different degrees of learning the instrumental act of escape. In this experiment, the escape response is drinking a morphine solution.

PROCEDURE

The experiment was conducted in a temperature-controlled room (72° F.). Thirty-nine female Sprague-Dawley rats weighing between 185 and 250 Gm. served as subjects. To establish motivation (withdrawal symptoms), daily intraperitoneal injections of morphine hydrochloride were started at 20 mg./Kg. and increased by equal increments to 160 mg./Kg. on the 29th day. Nine rats died during this period.

The remaining 30 morphine-tolerant, morphine-dependent rats were randomly assigned, 10 to group I and 20 to group II. Escape training cycles were then begun. In man, withdrawal distress reaches a peak forty-eight to ninety-six hours after the last opiate intake (12). Stanton (13) gives graphs which indicate that rats injected for four weeks show a peak of struggling when restrained during withdrawal which is similar to this time interval. Because of this, the escape training cycles were made three days long. Drinking the morphine solution reduced the current withdrawal symptoms and "re-set" the animal for a return of the symptoms three days later. One complete training trial (or cycle) consisted of twenty-four hours with no liquids (*O*), twenty-four hours with an 0.5 mg./cc. morphine hydrochloride solution (*M*), and twenty-four hours with tap water (*W*). Training proceeded as in this sequence (illustrating three trials and covering nine days): *O*, *M*, *W*, *O*, *M*, *W*, *O*, *M*, *W*, etc. The rats were individually caged and the solutions presented in 100-ml. graduated drinking tubes randomly located in the right or left positions on the front of the cage.

To test drug-seeking behavior, five choice tests were made in which two drinking tubes, one containing water and the other morphine solution, were presented simultaneously for a twenty-four hour period. Untrained rats refuse to drink the *M* solution, averaging 0.9 cc. under these conditions. For *M* consumption over 10 cc., the amount was read directly from the tube graduations. Amounts of *M* less than 10 cc. were measured by refilling the tube using a hypodermic syringe. Between the third and fourth, and between the fourth and fifth choice tests, the rats were removed from their individual, wire bottomed cages and placed in one large, straw litter-bottomed cage.

In previous experiments (3) it had been found that rats which drank less than 35 cc. of *M* on half or more of their training trials also drank very little (mean was 1.8 cc.) on their choice tests. In contrast, rats which drank more than this level averaged 13.6 cc. of *M* on their choice tests. This is consistent with the experimental design. A certain minimal morphine-intake is necessary to maintain tolerance, physical dependence, and thus, withdrawal symptoms from trial to trial during the training period.

Because of the small groups in this experiment, it was felt that these "minimal drinkers" could seriously reduce the precision of the experiment. Accordingly, a somewhat conservative rejection criterion was established. Rats which drank less than 20 cc. on half or more of their training trials continued to receive their assigned treatment and choice scores were obtained from them; but their scores were eliminated from statistical consideration when computing the main effects of the experiment.

All rats received the escape training treatment. In addition, the 20 rats in group II received three small morphine injections (2 mg. ea.) per cycle. Two injections were given on day (*O*) six and one-half and one and one-half hours before the beginning of day (*M*). The third injection was given on day (*W*) eight hours after the end of day (*M*).

After the first choice test, group II was randomly divided into two equal subgroups: IIA and IIB. Subgroup IIA continued the injection schedule given above for Group II. In IIB, the injection one and one-half hours before day (*M*) was eliminated. A new injection for IIB was then added one hour after the beginning of day (*O*). This new injection time was about midway between the two remaining original injection times. IIA and IIB continued these schedules to the end of escape training.

Due to a nonnormal distribution of scores, a nonparametric statistical technique, the Kruskal-Wallis one-way analysis of variance by ranks (11), was used.

RESULTS

Two rats (one each in IIA and IIB) died prior to the first choice test. Five rats (two in group I, one in group IIA, and two in group IIB) drank less than 20 cc. on half or more of their training trials and were eliminated by the rejection criterion. As a result, the final number of subjects was 8 in group I, 8 in group IIA, and 7 in group IIB.

The animals eliminated by the rejection criterion drank significantly less *M* than the nonrejected rats on all choice tests (p of 0.005, 0.01, 0.005, 0.01, and 0.01). The mean *M* intake for all choice tests for the eliminated rats was 2.6 cc.; that for the nonrejected rats was 12.3 cc. The mean oral intake of morphine on the training trials by the nonrejected rats was approximately 120 mg./Kg. per rat (Fig. 1).

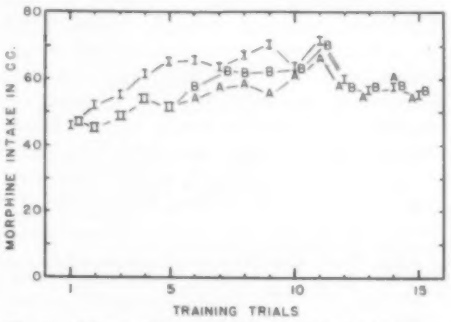

Fig. 1.—Mean consumption of morphine solution on 15 training trials.

Although IIA and IIB received small injections, neither the total morphine intake, nor the oral intake of *M* on the training trials (Fig. 1) differed significantly among the groups. Figure 4 gives the mean cc. intake of tap water on the choice tests. One difference, II *vs.* I for the second choice test, was significant at 0.05. Total liquid intake (tap water plus *M*) is given in Table I. Group differences in total liquid intake are not significant. Whether considered by groups or by combinations of groups, the following comparisons are nonsignificant; group differences in *M* intake on individual training trials, total morphine intake before each choice test, and total morphine intake for the experiment.

Figure 2 shows the mean oral intake of *M* by groups on choice tests (not training trials). The *M* intakes are significantly different when the combined injected groups are compared with the noninjected group (II *vs.* I). The significances of these differences on the first three choice tests are, respectively, 0.05, 0.005, and 0.05. This comparison approached significance (0.08) on the first abstinence choice test. No individual group comparisons were found to be significant; however, IIB showed the greatest mean morphine intake on all tests (Fig. 2), and had the only two rats which showed a highly pronounced relapse (Fig. 3). On the first abstinence test, one animal consumed 74 cc. of *M* (equivalent to 160 mg./Kg.), and another the equivalent of 98 mg./Kg. Even after twenty-nine more days of abstinence, the latter rat consumed 71 mg./Kg.

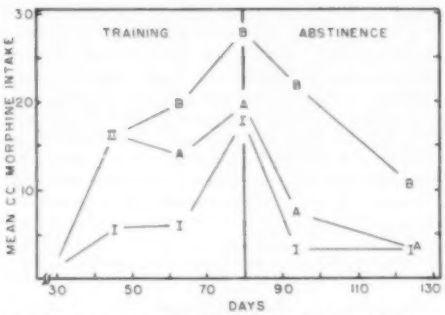

Fig. 2.—Mean consumption of morphine solution on 5 choice tests.

DISCUSSION

Consistent with previous findings (3), additional evidence indicates thirst is not an important factor in the procedure of this experiment. As morphine consumption increases, water intake decreases so that the total liquid intake remains essentially constant (Figs. 2, 4; Table I). There are no significant differences among the three groups in total liquid intake, nor for injected *vs.* noninjected groups (II *vs.* I).

The decrease in total liquid intake on choice tests four and five (during abstinence), as shown in Table I, reflects a change in availability of liquids. Rats, when put on a schedule which prohibits liquid intake one day out of three, increase their consumption on those days when liquids are available. Then, when transferred to an *ad libitum* drinking situation, this increase due to a restricted intake tends to disappear so that consumption approaches normal levels.

TABLE I.—MEAN CC. TOTAL LIQUID INTAKE BY GROUPS

Choice Test	I	IIA	IIB
1	65.1	65.8	67.9
2	66.5	58.8	60.4
3	76.5	57.7	70.8
4	43.0	44.3	47.2
5	49.8	57.1	50.0

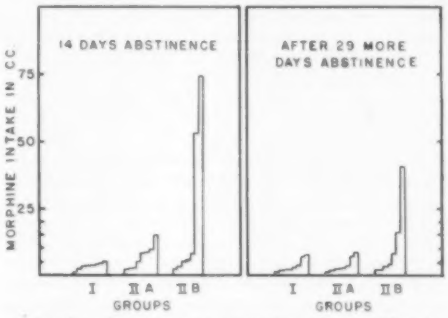

Fig. 3.—Graphic representation of morphine intake by individual rats on choice tests after periods of abstinence from morphine.

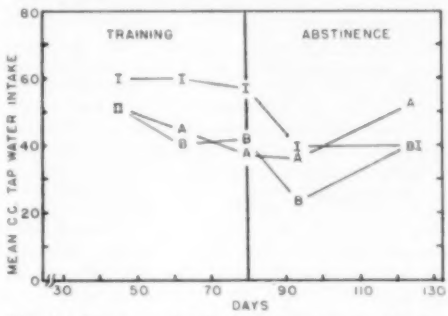

Fig. 4.—Mean cc. intake of tap water on the five choice tests.

Because tube positions were randomly determined during training and for choice tests, place learning cannot account for the results of this experiment. Neither are the results due to novel stimuli (3), nor can they be considered as artifacts produced by use of the rejection criterion. A comparison of the M intake of the injected groups with that of the noninjected group for all animals from which choice scores were obtained (N = 28), still gives significant differences of 0.01 and 0.025 for the second and third choice tests, respectively. Total morphine intake does not differ significantly for the same comparison. Also, the significant differences between the rejected and nonrejected rats indicate they may be justifiably considered as samples from different populations. It may be concluded then, that use of the criterion does not alter the general picture but, rather, provides a more precise measure of the experimental effects.

Before the first choice test, the effect of the injections given to Group II could not be predicted with any certainty due to the complexity of the situation. There is an intimate relationship among with-

drawal symptoms, physical dependence, and tolerance to the extent that it is possible to consider them as only different manifestations of one fundamental process (12). The question of effect of the injections was answered by the first choice test. The injected group was learning the response better than the noninjected group. A possible explanation of the results of this first choice test was that the injections acted to better maintain dependence and tolerance during the forty-eight hour interval between opportunities to drink the *M* solution. Because of this explanation, a new injection schedule for group IIB was established. This hypothesis, that dependence and tolerance were not well maintained during the intertrial period under the noninjection procedure, is consistent with the final results of the experiment. The 15 training trials used in this experiment did not noticeably increase *M* intake on the choice tests over that obtained with only 10 training trials used in previous experiments (3). An inefficiency in later training trials would be expected if dependence and tolerance were decreasing during the course of the experiment. Although all rats received increasing doses of morphine up to 160 mg./Kg. before the experiment and averaged more than 100 mg./Kg. of morphine per training trial, this apparently was insufficient to maintain a high level of tolerance and dependence through the later training trials.

If the injections given group II did, in fact, help maintain inter-trial dependence, then the new injection schedule for IIB could be expected to maintain inter-trial dependence even better since the injections were more evenly spaced for the inter-trial period. Maximum time without morphine on the original injection schedule was thirty-four hours. This was reduced to seventeen hours on the new schedule. The prediction of outcome at the time IIB was established was that group I would be low, IIA intermediate, and IIB highest in *M* intake on the choice tests. The fact that this order was obtained on all subsequent choice tests (Fig. 2); the form of the distribution of scores on the relapse tests (Fig. 3); and the two substantial relapses in IIB, all lend some support to the inter-trial maintenance-of-dependence hypothesis, although statistical significance could not be demonstrated for the differences between IIA and IIB.

The central finding of this experiment is that the injections did bring about significant increases in drug-directed behavior. Since differences in amount of morphine received cannot account for these increases, an explanation on some other basis must be advanced. It seems possible that the important difference between the injected and noninjected rats was a difference in their physiological state at the time morphine was presented. Some evidence on the interaction between physiological state and morphine intake is available in statements made by opiate addicts. They prefer to allow some withdrawal symptoms to develop before taking morphine. They try to explain this behavior by comparing it to hunger, "thus, 'eating a steak may always be enjoyable, but more so if one is hungry'" (12, p. 35). It appears possible, therefore, that the importance of the injections in the development of drug-seeking behavior in this experiment is due to the fact that they helped to better maintain and preserve, from trial to trial, the physiological state necessary for reinforcing the escape response, i.e., withdrawal symptoms. This explanation is consistent with the success of this and previous experiments (3) in producing drug-directed behavior, since the design of these experiments is based upon escape training and assumes the importance of withdrawal symptoms in reinforcing the escape response.

The most important conclusion to be drawn from this experiment is that the commonly supposed "inherent rewarding effect" of morphine is not sufficient to account for the results since differential effects were produced among the groups without significant differences in morphine intake. There were no significant differences in *M* intake on training trials, nor in total amount of morphine received before each choice test, nor in total morphine intake for the entire experiment. To hold that the results of this experiment depend on an "inherent rewarding effect" of morphine seems inconsistent with the fact that equivalent rewards for the three groups (since the amounts of "inherently rewarding morphine" did not differ significantly) produced unequal effects among the groups. The rats in control groups of previous experiments (3) also received at least as much morphine as the rats in their associated experimental groups but did not develop drug-seeking behavior. The difference produced in the behavior of these rats seems more closely related to the conditions under which morphine is received than it is to the amount of morphine received. This difference is similar to the difference in the behavior of man produced under different conditions of morphine intake (illegal users *vs.* hospital patients). It appears that for rats, as for man, the conditions under which morphine is received is a much more important factor in producing drug-directed behavior than the amount of morphine received. Thus, any explanation of drug-directed behavior in terms of some inherent rewarding properties of morphine seems insufficient to fully account for the drug-directed behavior of either rats or man.

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The Spectrophotometric Assay for Chlortetracycline[1] HCl and Tetracycline[1] HCl in Pharmaceuticals*

By FORTUNATO S. CHICCARELLI, MASON H. WOOLFORD, Jr., and MAURICE E. AVERY

Chlortetracycline heated under acid conditions forms anhydrochlortetracycline while tetracycline under the same conditions forms anhydrotetracycline. Anhydrochlortetracycline and anhydrotetracycline have similar spectrophotometric properties which make a distinction unreliable. Specific assays have been developed for chlortetracycline and tetracycline based on the formation of anhydro compounds and the fact that chlortetracycline heated at pH 7.5 is cleaved to isochlortetracycline while tetracycline under the same conditions is relatively stable.

SINCE ITS INTRODUCTION in 1948, chlortetracycline has been an important therapeutic agent in medical practice, while tetracycline (1, 2), the most recent of the tetracyclines, has become the most widely used broad spectrum antibiotic. Certification still requires the performance of a microbiological assay but this report will deal with the chemical assays currently in use by the Quality Control Section of Lederle Laboratories for the rapid and efficient control of production items.

Many of the fundamental chemical and physical properties of chlortetracycline serving as the basis for chemical assay have been described previously. Among these are paper strip chromatography (3, 4), polarography (5), nonaqueous titrimetry (6), fluorescence (7–9), and chemical color development (10–14). The assay of this compound in mixtures has also been proposed (17–19).

The chlortetracycline in the sample taken for analysis is brought into aqueous solution to a final concentration of approximately 100 μg./ml. Hydrochloric acid is added to one aliquot of the solution and the mixture is heated forming colored anhydrochlortetracycline which has an absorption maximum at 445 mμ. A second aliquot is heated at pH 7.5 and the chlortetracycline is cleaved to isochlortetracycline. Subsequent acid treatment does not rearrange the isochlortetracycline into anhydrochlortetracycline; thus the second aliquot can be used as the blank when calculating the chlortetracycline present.

Chemical methods suggested for chlortetracycline and oxytetracycline (10–16) for the most part are applicable to tetracycline because the three are related structurally. Paper strip chromatography (3, 4), polarography (5), and nonaqueous titrimetry (6) can also be adapted. Since tetracycline does not fluoresce under basic conditions, a fluorescence type assay similar to the one used for chlortetracycline (7, 9) cannot be adapted. Under basic conditions tetracycline solutions have a relatively stable color exhibiting a maximum at 380 mμ (20). The assay of mixtures has also been proposed (17–19).

The tetracycline in the sample taken for analysis is brought into aqueous solution to a final concentration of approximately 200 μg./ml. Two separate 5.0-ml. aliquots are heated with pH 7.5 buffer. The chlortetracycline is cleaved to isochlortetracycline while the tetracycline remains unaltered. One of the solutions is removed and cooled. After the addition of acid this solution is used as the blank. Acid is added to the second solution and heating is continued to form anhydrotetracycline. The second solution can be used as the assay sample. Blank and sample are measured at 434 mμ.

PHARMACEUTICALS CONTAINING CHLORTETRACYCLINE HCl

Specific Assay for Chlortetracycline

The spectrophotometric assay described in this report is a modification of the method of Levine, *et al.* (7), which appears in a volume by Grove and Randall (14), and is carried out according to the following scheme:

$$\text{chlortetracycline (tetracycline)}^{2} \xrightarrow[\text{HCl}]{\text{heat}} \text{anhydrochlortetracycline (anhydrotetracycline)}$$

Anhydrochlortetracycline (21) has an absorption maximum at 445 mμ while anhydrotetracycline has absorption maximum at 434 mμ. It is apparent that the above approach does not compensate for the presence of small concentrations of tetracycline

* Received August 25, 1958, from the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

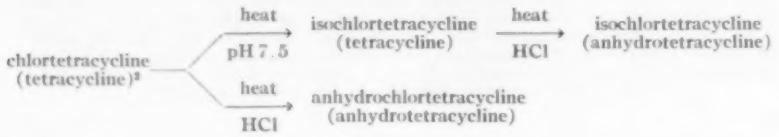
[1] Trademark of Lederle Laboratories Division, American Cyanamid Co., for chlortetracycline is Aureomycin and for tetracycline is Achromycin.

The authors wish to acknowledge with gratitude the kind help of Dr. C. W. Waller in the completion of this work, and to thank Dr. H. D. Piersma and Mr. P. Van Gieson for numerous suggestions.

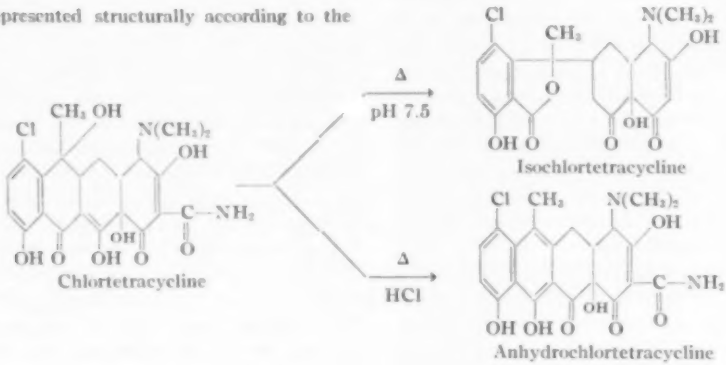
[2] Parentheses are used to show what would happen to low concentrations of tetracycline if present in chlortetracycline samples being assayed.

in chlortetracycline, which would be additive, and does not guarantee color production as coming from chlortetracycline only. The procedure of Hiscox (10) also uses this fundamental reaction but embodies measurements from different parts of the spectrum, and the assay is not as specific for chlortetracycline as that of Levine, *et al.*

A method previously described by Chiccarelli, *et al.* (22), eliminates these objections and is performed according to the following scheme:



This is represented structurally according to the following:



Oxytetracycline will not interfere with the above, since colorless apooxytetracyclines (23) are formed. Tetracycline because of its stability under basic conditions will form anhydrotetracycline both in the blank and sample. Since oxytetracycline is converted to colorless compounds in the blank and sample solutions and since tetracycline under the same conditions forms a mutually compensating color, the difference in absorbance between the blank and sample solution will result only from chlortetracycline.

Curve A of Fig. 1 is the absorption spectrum of chlortetracycline in 0.1 *N* hydrochloric acid; curve B is the absorption spectrum of isochlortetracycline under the conditions of the assay; and curve C is the absorption spectrum of anhydrochlortetracycline under the conditions of the assay.

Table I lists the concentration curve within the limits of the assay.

Table II lists time as a variable in the preparation of blank and sample solution. At the end of each time interval, hydrochloric acid was added and heating was continued in each case for a total of seven minutes. Thus on line 2 the solution was heated for two minutes at pH 7.5 then the acid was added and heating continued for five minutes more. It may be seen from Table II that seven minutes is optimum heating time for both the sample and the blank solution.

The assay is performed in the presence of sodium bisulfite which has a stabilizing effect on the background color during the pH 7.5 treatment.

Procedure for Application of This Assay to Pharmaceutical Products

Reagents.—Buffer solution pH 7.5.—178 Gm. of anhydrous K_2HPO_4 and 22 Gm. of anhydrous KH_2PO_4 per liter of water, filter before using; sodium bisulfite—10 Gm. per 100 ml. of water (freshly prepared); and hydrochloric Acid—5 *N*.

Procedure.—Final dilutions before acid and heat treatment should contain about 100 μg. of chlortetra-

TABLE I.

Concentration μg./ml.	Corrected Absorbance[a]	Absorptivity at 445 mμ
10.0	0.131	131.0
20.0	0.260	130.0
30.0	0.393	131.0
40.0	0.528	132.0
	Average	131.0

[a] Absorbance of sample solution minus absorbance of blank solution.

TABLE II.

Blank[a] Time, min.[b]	Blank[a] Absorbance at 445 mμ	Sample[a] Time, min.[c]	Sample[a] Absorbance at 445 mμ
1	0.070	1	0.017
2	0.025	2	0.050
3	0.010	3	0.088
4	0.006	4	0.117
5	0.004	5	0.127
6	0.004	6	0.134
7	0.004	7	0.134
		8	0.134
		9	0.131
		10	0.130
		11	0.125
		12	0.122

[a] Chlortetracycline standard initial concentration: 10 μg./ml.
[b] This column shows the time during which the solution was heated at pH 7.5.
[c] This represents the heat treatment while the solution was acidic.

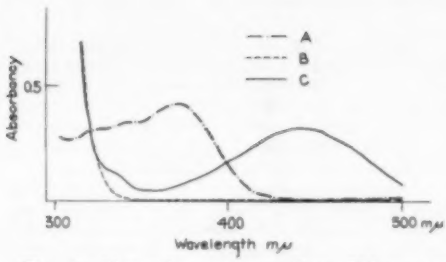


Fig. 1—Absorption spectrum from 300 mμ to 500 mμ of chlortetracycline, at 20 μg./ml. A in 0.1 *N* HCl; B under conditions of blank preparation; and C under conditions of sample preparation.

cycline per ml. See method of sample preparation.

Pipet a 10-ml. portion of the final dilution into each of two 50-ml. volumetric flasks. One of these will be the blank and the other the sample. To the sample add in this order: 12 ml. of 5 *N* HCl, 15 ml. of pH 7.5 buffer, and 2 ml. of bisulfite solution. Suspend in a boiling water bath for exactly seven minutes. Swirl occasionally.

To the blank add 15 ml. of pH 7.5 buffer solution and 2 ml. of sodium bisulfite solution. Suspend in a boiling water bath for five minutes with occasional swirling. After exactly five minutes add 12 ml. of 5 *N* HCl and heat for an additional two minutes.

After the completion of the heat treatment immediately cool both the blank and sample under tap water. Make to volume with water and mix well. Determine the absorbance at 445 mμ against water using a suitable spectrophotometer.

$$\frac{(A_{445}\text{ sample} - A_{445}\text{ blank})}{131.0^{3}} \times \frac{\text{dilution factor}}{\text{wt. of sample}} = \frac{}{\%\text{ chlortetracycline HCl}}$$

Sample Preparation

Capsules, Tablets, Powders, and Pharyngets[4].—Take an amount of sample equivalent to about 100 mg. of chlortetracycline. Transfer to a liter volumetric flask and add about 200 ml. of water. Mix until solution is complete. Make to mark with water. If filtration is necessary, use Whatman No. 42 paper. (Discard the first 10 ml. before taking an aliquot for assay.) Follow procedure.

Syrup, Pediatric Drops, and Cream.—Since calcium chlortetracycline is used in this type product, it will be necessary to add a small amount of hydrochloric acid in order to bring the chlortetracycline into solution. Transfer by weight or with a washout pipet a sample equivalent to about 100 mg. of chlortetracycline to a liter volumetric flask. Add 200 ml. of water and a small amount of dilute hydrochloric acid. Shake until solution is complete. Make to mark with water and mix well. Filter if necessary. Follow procedure.

Ointments.—Transfer a sample weight equivalent to about 100 mg. of chlortetracycline to a liter flask. Add exactly 100 ml. of CCl_4 and about 100 ml. of H_2O. Shake until sample is completely dispersed. Make to mark with water and mix well. Follow procedure. Do not consider the CCl_4 added as part of the dilution in the calculation. This fraction of the dilution will be 9 rather than 10.

Intravenous.—Weigh accurately about 150 mg. of powder. Transfer with the aid of 100 ml. of 0.1 *N* HCl to a liter flask. Make to mark with water and mix well. Follow procedure.

Spersoids[5] **Dispersable Powder.**—Weigh accurately about 3.0 Gm. of Spersoids. Transfer to a 500-ml. volumetric flask with about 100 ml. of water. Add 10 ml. of 1 *N* HCl and mix until all the lumps are dispersed. Make to mark with water and mix well. Filter through an S-1 filter pad on a Büchner funnel, discarding the first 100 ml. Follow procedure.

Troches.—Chlortetracycline troches contain a dye which interferes with the general spectrophotometric assay. In order to separate the dye from the chlortetracycline, a special modification of the spectrophotometric assay was developed. The assay is carried out in the following manner: Pulverize about five troches, accurately weigh the equivalent of about 30 mg. of chlortetracycline and transfer to a 250-ml. volumetric flask. Add about 100 ml. of water and mix until solution is complete. Make to mark with water and mix well. Using Whatman No. 42 filter paper, filter a portion of the sample. Discard the first ten ml. before taking a sample for assay. Pipet a 25-ml. aliquot into two glass-stoppered 50 ml. centrifuge tubes each containing 200 mg. of Magnesol magnesium trisilicate (Westvaco). The Magnesol should be slurried with water, filtered and dried before use. Shake for five minutes. Centrifuge. Decant the liquid carefully. Now add 15 ml. of water and mix well for three minutes. Centrifuge and decant the liquid.

Transfer each of the Magnesol and chlortetracycline complex samples to separate 100-ml. volumetric flasks with about 25 ml. of water. One of these will be the blank and the other will be the sample. To the sample add in this order: 24 ml. of 5 *N* HCl, 30 ml. of pH 7.5 buffer, and 4 ml. of bisulfite solution. Suspend in a boiling water bath for exactly seven minutes. Swirl occasionally.

To the blank add 30 ml. of pH 7.5 buffer and 4 ml. of bisulfite solution. Suspend in a boiling water bath for five minutes. This solution must be swirled constantly. After exactly five minutes have elapsed add 24 ml. of 5 *N* HCl and heat for an additional two minutes.

After completion of the heat treatment, immediately cool both the blank and the sample under tap water. Make to volume with water and mix well. Centrifuge a portion of the blank and sample. Determine the absorbance at 445 mμ with water in the reference cuvette. The absorptivity of chlortetracycline HCl under the above conditions of assay is 122.0. This value should be used rather than the one given in the general method of assay when calculating the amount of chlortetracycline per troche.

If available, a standard should be determined with each sample. This can serve as a check on the conditions of assay.

[3] Absorptivity of chlortetracycline HCl under assay conditions. A standard should be determined to ascertain the absorptivity under the particular conditions in each laboratory.

[4] Trademark of American Cyanamid Co.

[5] Trademark of American Cyanamid Co.

PHARMACEUTICALS CONTAINING TETRACYCLINE HCl

Specific Assay for Tetracycline

Tetracycline when heated under acid conditions forms anhydrotetracycline (21). An assay based on this reaction appears in a volume by Grove and Randall (14) and is carried out according to the following scheme (15):

$$\text{tetracycline (chlortetracycline)}^{a} \xrightarrow[HCl]{\text{heat}} \text{anhydrotetracycline (anhydrochlortetracycline)}$$

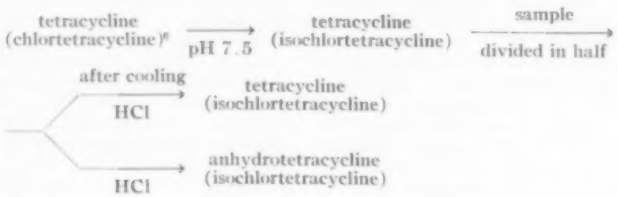
Anhydrotetracycline has a maximum at 434 mμ while anhydrochlortetracycline has a maximum at 445 mμ. The above approach does not distinguish between tetracycline and chlortetracycline and does not compensate for the presence of small concentrations of chlortetracycline in tetracycline.

A modification has been made of the method of Grove and Randall whereby the assay is made specific for tetracycline according to the following:

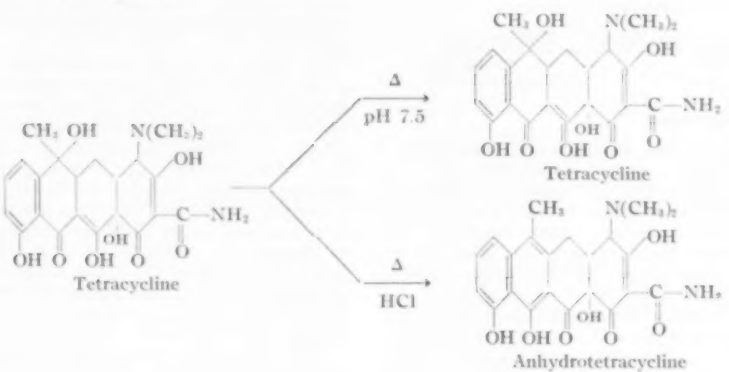
Curve A of Fig. 2 is the absorption spectrum of tetracycline in 0.1 *N* HCl; curve B is the absorption spectrum of tetracycline under the conditions of blank preparation; and curve C is the absorption spectrum of anhydrotetracycline under the conditions of the assay. Table III lists the concentration curve within the limits of the assay.

During the preparation of the sample, the solution is placed in a boiling water bath for three minutes at pH 7.5. Possible chlortetracycline interference is thus eliminated (7). Table IV lists time as a variable in the preparation of the sample solution. Each solution was heated at pH 7.5 for three minutes. Acid was added. After each time interval the solution was cooled and made up to volume. Five minutes is the optimum heating time for the sample solution after the addition of acid.

The assay is performed in the presence of sodium bisulfite which has a stabilizing effect on the background color.



This is represented structurally:



Tetracycline when heated at pH 7.5 is relatively stable and can be acid rearranged to anhydrotetracycline. Chlortetracycline, when heated at pH 7.5, forms isochlortetracycline which cannot be acid rearranged to anhydrochlortetracycline. Oxytetracycline, more stable under basic conditions, forms apooxytetracyclines (23) when heated with acid. Isochlortetracycline and apooxytetracyclines are colorless, so that any difference in absorbance at 434 mμ between blank and sample will result only from tetracycline.

[a] Parentheses are used to show what would happen to low concentrations of chlortetracycline if present.

Procedure for Application of This Assay to Pharmaceutical Products

Reagents.—Buffer solution pH 7.5.—178 Gm. of anhydrous K_2HPO_4 and 22 Gm. of anhydrous KH_2PO_4 per liter of water, filter before using; sodium bisulfite—10 Gm. per 100 ml. of water, freshly prepared; and hydrochloric acid—5 *N*.

Procedure.—Final dilutions before acid and heat treatment should contain about 200 μg. of tetracycline per ml. See method of sample preparation.

Pipet a 5.0-ml. portion of the final dilution into each of two 50-ml. volumetric flasks. To both flasks add 1 ml. of bisulfite solution and 10 ml. of

TABLE III.

Concentration μg./ml.	Corrected Absorbance[a]	Absorptivity at 434 mμ
5.0	0.081	162.0
10.0	0.161	161.0
15.0	0.243	162.0
20.0	0.320	160.0
25.0	0.402	160.0
	Average	161.0

[a] Absorbance of sample solution minus absorbance of blank solution.

TABLE IV.

Time, min.[a]	Corrected Absorbance at 434 mμ[b]
0	0.056
1	0.265
2	0.310
3	0.320
4	0.323
5	0.323
6	0.323

[a] This represents the heat treatment while the solution was acidic.
[b] Tetracycline standard initial concentration: 20 μg./ml.

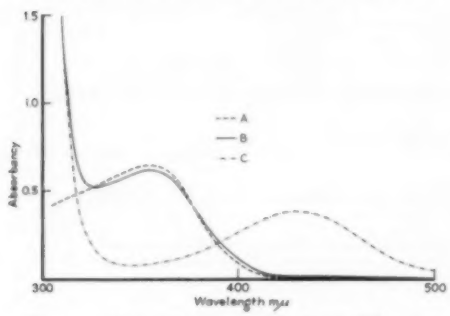


Fig. 2.—Absorption spectrum from 300 mμ to 500 mμ of tetracycline at 20 μg./ml. A in 0.1 *N* HCl; B under conditions of blank preparation; and C under conditions of sample preparation.

buffer solution. Place in a boiling water bath for exactly three minutes. Swirl occasionally during this period. After the three minutes have elapsed, remove one of the flasks and cool. This will serve as the blank.

To the remaining flask, add 6 ml. of 5 *N* HCl and heat for an additional five minutes with occasional swirling. This will be the sample.

After the blank has cooled, add enough water to give a total volume of about 40 ml. and add 6 ml. of 5 *N* HCl to the flask. Make to mark with water and mix well. When the sample has cooled, make to mark with water and mix well.

Determine the absorbance of the blank and sample at 434 mμ as soon as possible using water in the reference cuvette.

$$\frac{(A_{434}\text{ sample} - A_{434}\text{ blank})}{161.0^{7}} \times \frac{\text{dilution factor}}{\text{wt. of sample}} = \% \text{ tetracycline HCl}$$

[7] Absorptivity of tetracycline HCl under assay conditions. A standard should be determined to ascertain the absorptivity under the particular conditions in each laboratory.

Sample Preparation

Capsules, Powders, Tablets, and Pharyngets[4].—Take an amount of sample equivalent to about 100 mg. of tetracycline. Transfer to a 500-ml. volumetric flask and add about 200 ml. of water. Mix until solution is complete and make to mark with water. If filtration is necessary use Whatman No. 42 paper. Discard the first 10 ml. before taking an aliquot for assay. Follow procedure.

Suspensions, Syrups, and Pediatric Drops.—Since tetracycline neutral is used in this type product, it will be necessary to add a small amount of hydrochloric acid in order to bring the tetracycline into solution. Transfer by weight or with a washout pipet a sample equivalent to about 100 mg. of tetracycline to a 500-ml. volumetric flask. Add 200 ml. of water and a small amount of dilute hydrochloric acid. Shake until solution is complete. Make to mark with water. Filter if necessary. Follow procedure

Ointments.—Transfer a sample weight equivalent to about 50 mg. of tetracycline to a 250-ml. volumetric flask. Add exactly 50 ml. of CCl_4 and about 50 ml. of H_2O. Shake until sample is completely dispersed. Make to mark with water and mix. Follow the procedure. Do not consider the CCl_4 added as part of the dilution in the calculation.

Spersoids[5] Dispersible Powder.—Transfer a 3-Gm. sample (50 mg. of tetracycline) with the aid of water to a 250-ml. volumetric flask. Add 10 ml. of 1 *N* HCl and mix until all lumps are dispersed. Make to mark with water and mix well. Filter through an S-1 filter pad on a Büchner funnel, discarding the first 75 ml. Follow procedure.

Oil Suspension.—Using a washout pipet, transfer a 5.0-ml. sample (50 mg. of tetracycline) to a 250-ml. volumetric flask. Wash the pipet with acetone (25 ml.). Mix. Add slowly and with constant swirling, 20 ml. of 1 *N* HCl and about 100 ml. of water. When the oil has coagulated, dilute to mark with water and mix. Filter through Whatman No. 42 paper, discarding the first 10 ml. Follow procedure. Do not consider the oil as part of the dilution in the calculation.

Troches.—Use the isolation and purification technique described under chlortetracycline troches. After this, transfer the Magnesol magnesium trisilicate residues containing tetracycline to separate 100-ml. volumetric flasks with about 25 ml. of water. One of these will be the blank and the other the sample.

Now add 1 ml. of bisulfite solution and 10 ml. of buffer solution to each flask. Suspend in a boiling water bath for four minutes. Swirl occasionally. After the four minutes have elapsed, remove one flask and add 6 ml. of 5 *N* HCl to the other. Cool the one removed, make to about 90 ml. with water, add 6 ml. of 5 *N* HCl then dilute to mark and mix well. This will be the blank.

After adding 6 ml. of 5 *N* HCl to the flask in the boiling water bath, heat for an additional five minutes. Cool and make to mark with water. This will be the sample.

Centrifuge a portion of the blank and sample. Determine the absorbance at 434 mμ with water in the reference cuvette. The absorptivity of tetracycline HCl under the above conditions of assay is 155.0. This value should be used rather than the

one given in the general method of assay, when calculating the amount of tetracycline per troche.

If available, a standard should be determined with each sample. This can serve as a check on the conditions of assay.

SUMMARY

1. Specific spectrophotometric assays have been developed for tetracycline and chlortetracycline.

2. Procedures are described in which the assays may be used for various types of pharmaceutical products.

3. An isolation technique has been developed that may be adapted to other problems.

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Polarography of Some Phenylmercury Amide and Imide Compounds*

By SISTER M. LAWRENCE O'DONNELL and CORNELIUS W. KREKE

Several amide and imide compounds of phenylmercury were prepared and their stability studied polarographically under different conditions. The half-wave potentials of these compounds were found to be identical, within experimental error, to the decomposition potential of phenylmercuric hydroxide, suggesting a ready hydrolysis in each case. Other characteristics of the polarograms were also compared. Polarograms of mixtures of the coordinating compounds with phenylmercuric hydroxide in molar ratios greater than 1:1 were obtained. An increase in the half-wave potentials to greater negative values in the case of the amides of higher molecular weight and also succinimide indicates a greater binding of these compounds with phenylmercury as contrasted with the amides of lower molecular weight which did not shift the half-wave potentials under the conditions employed.

IN A RECENT PUBLICATION by Smalt, *et al.* (1), several phenylmercury compounds were studied as biological inhibitors with oxidative enzymes as test systems. The authors found that with the exception of phenylmercuric succinimide and phenylmercuric phthalimide all the compounds studied were quantitatively similar to phenylmercuric hydroxide, and concluded that the compounds dissociated readily in water presumably acting on the enzymes as the free phenylmercuric ion.

The mode of action of the organic mercurials is assumed to involve a specific reaction with sulfhydryl groups of the proteins of the cell (2). Still greater specificity is also postulated by Hughes (2), selective binding sites among the sulfhydryl groups resulting in differences in biological action as diuretics, germicides, fungicides, antiseptics, and antisyphilitics. The situation is complicated, however, since some enzymes which are normally considered non-sulfhydryl dependent are also inhibited by the mercurials (3, 4) suggesting that in these cases the phenylmercuric ion or the undissociated molecule reacts nonspecifically with other essential groups.

Since the phthalimide compound and to a lesser extent the succinimide compound were not as effective as phenylmercury hydroxide in the depression of enzyme activity, the inference can be drawn that they are less readily hydrolyzed,

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an inference which seems strange in view of the fact that phenylmercury amide compounds were among the most effective as inhibitors.

In order to test this hypothesis, several phenylmercury amide and imide compounds were prepared and tested polarographically for their stability in water solution. Comparisons were made with the parent compound, phenylmercuric hydroxide, in the magnitude of the half-wave potentials.

EXPERIMENTAL

Preparation of Compounds.—Equimolar mixtures (0.01 moles) of the phenylmercuric hydroxide (Berk & Co.), recrystallized from water and the amide or imide (Eastman) compounds were placed in a flask and 100 ml. of water added. This mixture was refluxed for thirty minutes and the solid precipitate filtered off and recrystallized from water or an alcohol-water mixture. The compounds were dried in the vacuum desiccator and analyzed for mercury by the method of Pierce (5). The compounds prepared are given in Table I along with their analyses and melting points.

TABLE I.—MELTING POINT AND ANALYSIS OF SOME PHENYLMERCURY COMPOUNDS

Compounds	Mercury, %—Calcd.	Mercury, %—Found	Melting Point Uncorrected, °C.
PM hydroxide[a]	...	.....	224–227
PM acetate[a]	...	.....	147–150
PM acetamide[a]	...	.....	160–162
PM propionamide	57.35	57.54–56.09	95–96
PM butyramide	55.14	57.79–55.54	66–67
PM isobutyramide	...	.....	69–70
PM valeramide	53.09	52.05	74.5–75.5
PM isovaleramide	53.09	52.10–52.34	78–81
PM hexanamide	...	.....	60–62
PM nonanamide	46.22	46.06	72.5–73.5
PM benzamide[a]	...	.....	172–173
PM succinimide[a]	...	.....	195–196.3
PM phthalimide[a]	...	.....	212.5–213.5

[a] Compound not new.

Polarography.—The Polarograph used was a Sargent Manual Model III. The potentials were measured against the standard saturated calomel electrode. At a pressure of 60 cm. of mercury the drop time (t) was 3.2–3.6 sec./drop. The Lingane and Laitinen (6) H-cell was used. Dissolved oxygen was removed by passing nitrogen through the test solution for 15 minutes. The supporting electrolyte was 0.05 N $KClO_4$. Three drops of 0.01% gelatin were used to suppress the maxima.

Preparation of Test Solutions.—Stock solutions of the phenylmercury compounds were made up in water at a concentration of 12×10^{-4} M except in the case of the phthalimide compound where decreased water solubility made it difficult to work with levels above 4×10^{-4} M. Fifty milliliters of the stock solution and 100 ml. of 0.1 N $KClO_4$ were placed in a 200-ml. volumetric flask, the pH adjusted to 7.0 with 0.1 N KOH and the solution diluted to the mark (final concentration 3×10^{-4} M). It was found that during the polarographic reduction there was a change of not more than 0.2 pH toward the acid side.

Results and Discussion.—All of the phenylmercury compounds studied were reductively decomposed in a two single electron step process similar to that demonstrated before by Benesch and Benesch (7) and Wuggatzer and Cross (8) for similar mercury compounds. According to Benesch and Benesch (7) the two waves correspond to the two step reduction:

$$RHgOH + H^+ + e \ldots RHg^\bullet + H_2O$$
$$RHg^\bullet + H^+ + e \ldots RH + Hg$$

In the first step which occurred at a voltage of 0.200–0.220 the hydroxide or phenylmercuric ion is reduced to the phenylmercury radical. In the second step, the radical is decomposed into the hydrocarbon and free mercury ($E^1/_2 = -1.14$ v.).

This reduction is reported to be irreversible (6) and one might expect it to be so since the phenylmercury radical is formed. It was reported by Benesch and Benesch (7) that the slopes of the waves varied with conditions such as concentration and pH and no simple relationship between potential and current (such as a linear plot of E *versus* log $i/(id - i)$) was found to describe the waves. Also the phenylmercury radical and resulting reaction products are absorbed in the mercury surface and lead to some wave irregularities. However, according to Lingane (9) the half-wave potential is a characteristic of a compound even though the decomposition is not reversible and Calvin and Bailey (10) on this basis were able to make a comparison of the stability of a whole series of copper chelates.

In this work a comparison was made of the half-wave potentials of some phenylmercury amide and imide compounds. It was found that all of the compounds were reductively decomposed in a two-step process similar to that of the parent compound and the data reported here give the range of potentials obtained. The work of Benesch and Benesch (7) and Wuggatzer and Cross (8) showed that there is considerable variation in the half-wave potentials of phenylmercury compounds with change in buffer, pH, and concentration of the mercurial. For instance, Wuggatzer and Cross report values for the first half-wave potential for phenylmercuric nitrate as −0.132 v. at pH 7.0 in Britton-Robinson buffer and −0.247 v. in Michaelis buffer system. Under the condition of our experiments we obtained the results shown in Table II. The value of the first half wave for phenylmercuric hydroxide varied from −0.20 to −0.22 v. with an average of 0.210 v. Among the phenylmercury amide and imide compounds there was somewhat more variation in the first half wave. Values obtained varied from 0.200 v.

for the phenylmercury benzamide and phenylmercury phthalimide to −0.245 v. for the isovaleramide and succinimide compounds. Since the averages for the half-wave potential for each compound were approximately the same as that of the phenylmercuric hydroxide and considering the overlapping in the range of values obtained for each compound it was concluded that not much significance could be attached to these variations. Accordingly, it is apparent then that these mercury amide and imide compounds dissociate readily in water to the hydroxide so that in each case the reductive decomposition of phenylmercuric hydroxide is measured.

This comparative study was repeated under a variety of conditions such as lower concentration of mercury compound, other carrying electrolytes as potassium acetate, and with buffer systems such as that prepared by Britton-Robinson. The same relative results were obtained but the potentials were shifted to lower values (−0.10 to −0.18 v. for the hydroxide) and the reproducibility of data was not as good. With potassium acetate as carrying electrolyte, high residual currents were obtained, probably because of the formation of mercuric acetate complexes. The results obtained in Britton-Robinson buffer agreed well with those of Benesch and Benesch (7) and Wuggatzer and Cross (8). A slight increase in the half-wave potentials was obtained for all the compounds with an increase in pH, suggesting the irreversibility of this reduction (7).

The second half wave of phenylmercury hydroxide was −1.145 v., an average of ten determinations. With the phenylmercury amide and imide compounds, there was considerable variation in this second half wave, values obtained under the condition of our experiments varying from −1.080 in the case of the propionamide compound to −1.225 v. for the valeramide compound or a range of 145 mv. Since this potential measures the same reaction in all cases, that is, the reductive decomposition of the phenylmercury radical, less variation might be expected. The overlapping of the ranges of values obtained suggests that the variations are not significant.

Phenylmercury hydroxide had an average wave height of 1.6 microamp. in the first wave and 3.61 microamp. in the second. This agrees with the average wave heights for the amide and imide compounds of 1.56 (range 1.48–1.93) and 3.83 (range 3.32–4.19 microamp.). The wave heights increased for all the compounds with increasing concentration of the mercurial but varied with the different buffers used and the acidity of the media.

In Table III is given data showing the effect of increasing concentration of the amides and imides on the half-wave potential of the first reductive decomposition of phenylmercuric hydroxide. It will be observed that with the amides of lower molecular weight there is no change in the half-wave potential even in molar ratios of amide to mercurial of 1,000:1. With the hexanamide there is a significant increase in negative potential (range −0.300 to −0.342 v.) at a molar ratio of amide to mercurial of 1,000:1 amounting to an average increase of 111 mv. A significant increase was also obtained with succinimide in a molar ratio of 100:1 (range of potential −0.255 to −0.262 v.) and for nonanamide in a molar ratio of 10:1. The effect of nonanamide above this ratio could not be studied because of its insolubility in water.

TABLE II.—FIRST HALF-WAVE POTENTIALS FOR PHENYLMERCURY AMIDE AND IMIDE COMPOUNDS

Compound	First Half-Wave Potential[a]
PMOH	0.200–0.220
PM acetamide	0.212–0.225
PM propionamide	0.212–0.240
PM valeramide	0.216–0.235
PM isovaleramide	0.226–2.245
PM hexanamide	0.200
PM nonanamide	0.220–0.225
PM benzamide	0.200–0.214
PM succinimide	0.220–0.245
PM phthalimide	0.200–0.220

[a] At least 10 determinations were made, in some cases 20.

Since the excess of amides and imides did not change the ionic strength of the media, nor apparently change the wave heights of the first or second wave and did not significantly change the potential of the second reductive decomposition it must be concluded that the effect of the hexanamide, nonanamide, and succinimide (and presumably phthalimide) was one of shifting the hydrolytic equilibrium toward the complex. How far the equilibrium is shifted is, of course, impossible to ascertain from these experiments. The lack of appearance of a third wave, one for the decomposition of the phenylmercuric amide or imide compound as was found by Benesch and Benesch (11) for glutathione and phenylmercury may indicate either that the position of this third wave is sufficiently close to that of the phenylmercuric hydroxide so that only a resultant wave was obtained under the condition of our experiments or that the shift of equilibrium by the excess of amides and imide was relatively small even with the large excess of complexing compound.

In either case it is evident that the amide compounds of lower molecular weight are readily hydrolyzed even with the amides present in large excess. This is also true for the amides of higher molecular weight and also the imides when present in solution with the phenylmercuric hydroxide in a molar ratio of 1:1. A rough parallelism seems to exist between the increase in negative half-wave potential and molecular weight of the complexing amide or imide, with the increase in organic solubility of the resulting phenylmercury amide and imide compound, and also with their decrease in water solubility.

DISCUSSION

K. L. Mandal studied the formation and stability of 3- and 4-coordinated compounds of mercury dibenzidine (12) and also 4-coordinated mercuric salts with diamines (13). He concluded from his work that mercury does not appear to form stable 4-coordinated compounds, hydrolyzing very readily in water to the more stable 3-coordinated compound. The one exception he found was the dibenzidine dipiperidine compound which was insoluble in water. Among the 3-coordinated compounds only the dibenzidine pyridine and piperidine compounds were stable in water. Diamines both aliphatic

TABLE III.—EFFECT OF EXCESS CONCENTRATIONS OF AMIDES AND IMIDES ON THE HALF-WAVE POTENTIAL OF PHENYLMERCURIC HYDROXIDE

	Molar Ratios[a]			
	3×10^{-4} 1:1	3×10^{-3} 1:10	3×10^{-2} 1:100	3×10^{-1} 1:100
Acetamide	−0.219	−0.217	−0.217	−0.226
Propionamide	−0.216	−0.217	−0.214	−0.207
Valeramide	−0.220	−0.214	−0.203	−0.225
Hexanamide	−0.213	−0.218	−0.244	−0.326
Nonanamide	−0.232	−0.364 −(0.350–0.440)	.....	−(0.300–0.342)
Succinimide	−0.222	−0.225	−0.258 −(0.255–0.262)	−0.270 −(0.250–0.280)
Phthalimide	−0.202	.....	.....	.....

[a] At least 9 determinations for each.

and aromatic appear to form 4-coordinated compounds which are stable in organic solvents.

Our work, in general, confirms that of Mandal. While it is comparatively easy to prepare the 2-coordinated compounds of phenylmercury such as the phenylmercuric amide and imide compounds, attempts to prepare amide and imide compounds in which the coordination was 3 and 4 were unsuccessful. Reactions were attempted by heating in several organic solvents ($CHCl_3$, acetone, alcohol, ether) and also by melting the compounds together. The 2-coordinated compound was obtained on crystallization.

Preparation of phenylmercury compounds of bi and tridentate chelating agents such as glycine, β-alanine, γ-aminobutyric acid, ethylene diamine, diethylene triamine, glycylglycine, and EDTA (see table IV) were successful but no evidence could be obtained polarographically that chelation took place. EDTA added to a phenylmercuric hydroxide solution in molar ratios of 1:1 and 10:1 had a $E^1/_2$ of 0.240 or about 0.02 v. more electronegative than phenylmercuric hydroxide, thus indicating only a slightly greater stability in water solution. However, in a 50:50 (v/v) pyridine water solution with 0.1 *M* potassium nitrate as carrying electrolyte, phenylmercuric γ-aminobutyric acid had a $E^1/_2$ about 375 mv. more negative than phenylmercuric hydroxide.

TABLE IV.—MELTING POINT OF SOME POSSIBLE PHENYLMERCURY CHELATE COMPOUNDS

Compound	Melting Point, °C.	Possible Coordination of Resulting Compound
PM β-alanine	195–215d	3
PM γ-aminobutyric acid	166–170d	3
PM glycine	120–124	3
PM glycylglycine	162–163d	4
PM diethylene triamine	122–123	4
PM EDTA	....	4

While phenylmercury may form stable chelates in organic solvents it is evident that among the compounds studied the complexes of phenylmercury are readily hydrolyzed as shown from this study. The ready dissociation of all the amide and imide compounds observed in this study partially confirm the conclusions of Smalt, *et al.* (1), who found that phenylmercuric acetamide and benzamide were equally as effective as phenylmercuric hydroxide as biological inhibitors and who also found a ready dissociation of these compounds in chromatographic studies with neutral solvents. The results of this study are not consonant with their conclusion as to the lesser dissociation of the phenylmercuric succinimide and phthalimide compounds.

From these polarographic studies it is evident that the decreased biological activity of the phthalimide and succinimide cannot be explained on the basis of greater stability. Possibly a greater lipoid solubility or a decreased water solubility may play a part in determining the germicidal efficacy. It was observed in this study that the formation of the amide and imide complexes greatly decreased the solubility of the phenylmercury in water and increased its solubility in organic solvents and it was noticed also that this solubility effect increased with increasing molecular weight of the complexing ligand.

From the data presented it seems that the stability of the phenylmercuric imides is no greater than that of the amides unless one compares the imides with the amides of lower molecular weight. This observation is only partially in agreement with the findings of Whitmore (14) from measurements of conductivity that the Hg—N linkage of imides is more stable than the Hg—N of amides.

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Studies on Antibacterial Vapors of Volatile Substances*

By THOMAS C. GRUBB

Two simple methods are described for making a quantitative estimate of the bacteriostatic and bactericidal action of vapors from volatile substances. One method is used for static exposure periods of twelve to eighteen hours, the other is for dynamic exposure periods as short as one second. The results of screening twenty-two compounds by the former method are described. The types of bacteria commonly associated with respiratory infections were employed as test organisms. It is interesting to observe that many of the drugs which have been employed empirically many years for the inhalation therapy of respiratory infections display a measurable degree of *in vitro* antibacterial and antiviral activity.

FOR CENTURIES mankind has sought to alleviate and cure its sufferings from many types of diseases by the inhalation of vapors from volatile oils, incense, etc. It seemed reasonable to believe that if the disease was localized in the respiratory tract, or if the most severe symptoms were confined to this area, the most direct attack on the source of the disease was by the inhalation of some volatile medication. As empiricism gave way to the scientific method, bacteriologists began to test the volatile substances which folklore had credited with therapeutic value. Thus within a comparatively short time, a number of publications appeared which clearly demonstrated by accepted techniques that the vapors from a number of substances were able to inhibit the growth or actually kill many different types of pathogenic bacteria and some viruses. In 1920 Macht and Kunkel (1) showed that the vapors from myrrh, cinnamon, incense, etc., exhibited distinct bacteriostatic but slight bactericidal action on *Escherichia coli* and *Pseudomonas pyocyaneus*. Morel and Rochaix (2) exposed meningococci, typhoid bacilli, staphylococci, diphtheria bacilli, and anthrax spores to the vapors of various essential oils. Of these, the oils of citron, thyme, and orange were found to be germicidal after fifteen minutes contact for some species while other species required as long as eight hours exposure. However, they were unable to kill anthrax spores under any of their experimental conditions. Schöbl and Kusana (3) showed that vapors from a number of essential oils had a bacteriostatic effect on the tubercle bacillus, *Vibrio cholerae*, and staphylococci. Walker, *et al.* (4), noted that vapors from onions possessed bactericidal activity. In 1927 Wrenn (5) irradiated cod liver oil, cottonseed oil, olive oil, etc., and concluded that secondary germicidal emanations were produced from these oils. However, five years later, Harris, *et al.* (6), demonstrated that Wrenn had incorrectly interpreted his findings since the germicidal action of the irradiated oils resulted from the production of volatile peroxides. These same workers (7) also studied the effect of irradiating various fish and vegetable oils in the production of antiseptic vapors. After a fifteen-minute irradiation period, cod liver oil vapors inhibited *Streptococcus epidemicus*, *Eberthella typhosa*, *E. coli*, etc. Moreover, if these oils were irradiated for twenty-five minutes, then their vapors prevented the germination of *Clostridium sporogenes* and *Bacillus subtilis* spores. The antibacterial activity of garlic vapors was studied by Walton and his co-workers (8) who reported that expsoure for periods of four to one hundred twenty minutes at 37° was inhibitory for *Mycobacterium butyricum*, *Mycobacterium smegmatis*, and *Serratia marcesens*. In another publication by McKnight and Lindegren (9), *Mycobacterium leprae* was inhibited after one hour's exposure to garlic vapors at 37°. The germicidal activity of these vapors was attributed to their acrolein content by Vollroth, *et al.* (10).

In 1940 Lebduska and Pidra (11) published the most extensive study on the antibacterial action of vapors reported in the literature up to that point. These workers streaked plates with *E. coli* and *S. aureus* and exposed them to the vapors of a variety of substances, many of which displayed bacteriostatic or bactericidal action. In the same year Foter (12) tested the antibacterial activity of vapors from allyl isothiocyanate and related oils. He concluded that the bacteriostatic action of garlic vapors was due to their allyl isothiocyanate content.

The vapors from cinnamon and eucalyptus oils were found by Remlinger and Bailly (13) to be bactericidal for the typhoid and paratyphoid bacilli as well as neutralizing the viruses of rabies, pseudorabies, and equine encephalo-

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TABLE II.—COMPARATIVE ANTIBACTERIAL VAPOR

myelitis. In China, Chu and Pai (14) described the bacteriostatic action of garlic vapors for *Staphylococcus aureus* after ten minutes contact and *E. typhosa* after twenty minutes contact at 37°. In Russia, Tokin (15) studied the antibacterial action of vapors from various plants and concluded that the vapors from onion and garlic were the most potent. Tokin found that an onion paste ceased to produce bactericidal vapors ten to fifteen minutes after it was prepared. The mechanism of the antibacterial vapors from cod liver oil was further clarified by Ross and Poth (16) who demonstrated that fresh oil did not produce antiseptic vapors against *S. aureus*, *B. subtilis*, and *E. coli*, but that oil which had been exposed to the air or had its natural antioxidants removed did give off antiseptic vapors. Their results suggested that the germicidal principle in the vapors was acrolein.

EXPERIMENTAL

The purpose of this report is to describe *in vitro* methods for making a quantitative evaluation of the antibacterial activity of the vapors of volatile compounds. Attention was directed primarily to compounds which might be used therapeutically rather than to substances which are used on inanimate objects, e. g., ethylene oxide, beta-propiolactone, triethylene glycol, etc.

When it is not important to determine how rapidly vapors inhibit or kill bacteria, a static technique may be used as follows: Exactly 20 cc. of nutrient agar are poured into Petri dishes, and after the medium has solidified, one drop of a 1:100,000 dilution of *S. aureus* is placed on the agar and spread uniformly over the surface with a sterile glass rod. The inoculated medium is then inverted over the top of the cover of the Petri dish which contains the substance to be tested. There are a number of ways that the vapors from the test substance may be evaluated, however, it is important to remember that where quantitative tests are being carried out for comparative or control purposes, it is essential that the quantity and area of the exposed surface of the test substances be identical in all tests. Best results have been obtained by annealing glass rings 20 mm. in diameter and 0.5 mm. high in the exact center of the inner surface of a Petri dish top. The substance to be tested is then placed in this cup and the inoculated bottom inverted over it. Less satisfactory is the application of the test substance to a filter paper circle approximately 4 mm. in diameter which is placed in the center of the dish cover. If the test substance is a powder, it may be dissolved in some nonvolatile solvent, the filter paper then dipped into it and placed on the inner surface of the Petri dish top. Volatile solvents may be used provided a control with the solvent alone is tested, and if it also produces antibacterial vapors, the effect of these vapors must be taken into account in determining the net effect of the compounds under test.

Since a considerable variation in plate counts is to be expected, it is essential to employ a sufficient number of replicate plates so that the results will be statistically significant. After the plates have been prepared, they are placed in the 37° incubator for eighteen to twenty-four hours before counting. The covers are then removed and replaced with fresh sterile tops not containing the test substance. These plates are then returned to the incubator for an additional eighteen to twenty-four hours. The first count may be considered the "bacteriostatic" count and the second, the "bactericidal" count, since colonies often appear after forty-eight hours which were repressed during the initial incubation period. One additional test may be carried out to be certain that the absence of growth after the forty-eight hours incubation period is the result of bactericidal action and not merely bacteriostasis caused by absorption of the vapors into the medium. A sterile cork borer 1.0 cm. in diameter is used to remove agar cores from the plates where no growth has developed (while the control plates show considerable growth in a corresponding area). Each core is transferred aseptically to a flask containing 250 cc. of nutrient broth and incubated at 37° for one week. If no visible growth develops, it is reasonable to assume that no viable bacteria were present in the agar sample tested. Using the above methods, many variations may be introduced according to the needs of the problem. For example, different organisms, media, and concentrations of the inoculum may be employed. Table I shows not only the bacteriostatic and bactericidal action of 0.5% chlorothymol mixed with other essential oils, but also indicates a vast difference in the resistance of two different strains of *S. aureus* to these vapors.

TABLE I.—BACTERIOSTATIC AND BACTERICIDAL ACTION OF CHLOROTHYMOL VAPORS ON *S. aureus*

Test Substance	Total Number of Colonies per Plate			
	24 Hr. Incubation		48 Hr. Incubation	
	M Strain	B Strain	M Strain	B Strain
Control	265	330	265	354
	222	352	225	360
	252	293	260	300
	93	308	93	310
	225	...	225	...
Mean	211	321	213	331
0.5% Chlorothymol	259	0	270	4
	246	0	246	13
	212	0	220	4
	232	0	235	0
	280	0	280	8
Mean	246	0[a]	250	6[a]

[a] Agar plugs cultured from areas without colonies did not show any growth after seven days incubation at 37°.

In Fig. 1 the number of colonies and their distribution when a resistant and susceptible strain of *S. aureus* are exposed to the vapors of 0.5% chlorothymol for twenty-four hours may be observed.

Using the static technique, over 50 different substances were screened for their antiseptic vapor action, with *S. aureus* and *Klebsiella pneumoniae* as the test organisms. In Table II only those substances which showed some activity against either of the test organisms are recorded.

These results indicate that many more substances are active against *S. aureus* than against *Kl. pneumoniae*, and if one substance is effective against both

TABLE III.—ANTIBACTERIAL ACTION OF OXYQUINOLINE VAPORS ON VARIOUS RESPIRATORY BACTERIA

TABLE II.—COMPARATIVE ANTIBACTERIAL VAPOR ACTION OF VARIOUS SUBSTANCES ON *S. aureus* and *Kl. pneumoniae*

Inhibition of Growth, %	Test Organism	Substance[a]
75–100	*S. aureus*	Salicylaldehyde
		Oxyquinoline
		Chlorothymol
		Oil of mustard
	Kl. pneumoniae	Oxyquinoline
25–75	*S. aureus*	Thymol
		Lauryl alcohol
		Phenyl isothiocyanate
	Kl. pneumoniae	None
25–50	*S. aureus*	Oxyquinoline benzoate
		Methyl cinnamate
		Caproic acid
		Turpentine
	Kl. pneumoniae	Oxyquinoline benzoate
		Salicylaldehyde
5–25	*S. aureus*	Oil of cloves
		Oxalidine
		Menthol
		Chlorobutanol
		Beta pinene
		Decyl alcohol
		Camphene
		Oleoresin of capsicum
	Kl. pneumoniae	Chlorothymol

[a] Dilutions of 1–5% in triethylene glycol or petrolatum.

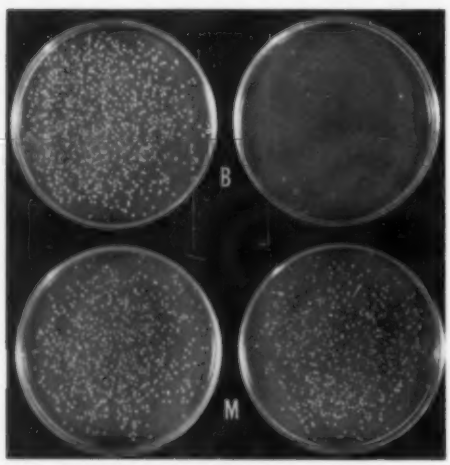

Fig. 1.—Comparative resistance of *S. aureus* strains. Number of colonies developing in control (left) and 0.5% chlorothymol (right) static vapor test after twenty-four hours exposure. Note difference between susceptible B strain (upper) and resistant M strain (lower).

organisms, it is usually more effective against *S. aureus*.

In addition to the testing of antibacterial vapors under static conditions, it may be important to test them under dynamic conditions in which the vapors pass rapidly across the surface of the test organisms for various time intervals. The apparatus employed for this purpose is shown in Fig. 2.

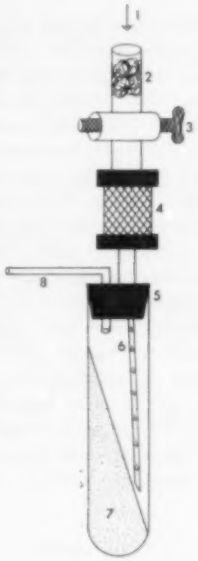

Fig. 2.—Diagram of apparatus employed in the dynamic vapor exposure tests. 1, air inlet; 2, cotton air filter; 3, stopcock; 4, gauze impregnated with test substance; 5, two-hole rubber stopper; 6, copper tube with pinholes; 7, agar slant; and 8, air outlet.

Through one hole of a two-hole rubber stopper is passed a copper tube (0.2 mm. diam.) 10 cm. long closed at the distal end and having "pin" holes (0.1 mm. diam.) every 2 cm. along the length of the tube; and through the other hole is passed a glass tube 5 mm. in diameter. The stopper is inserted tightly into the end of a test tube containing an inoculated agar slant. Sterile air is drawn through the tube containing cotton gauze impregnated with the substance to be tested and into the copper tube inserted into the culture tube in such a way that the pin holes are facing the agar slant, and the air flows uniformally across the inoculated surface of the agar. The outlet tube is connected to a flowmeter. The air flow is adjusted to 17.5 cc./second and by means of the stopcock, the inoculated medium can be exposed to vapors for any time interval from one second upward.

After the inoculated tube has been treated with the flowing vapors for the desired period, it is removed from the two-hole stopper and the sterile cotton plug replaced with aseptic precautions. After all the tubes in a given experiment have been exposed to the vapors, they are incubated at 37° for twenty-four hours and the number of developing colonies counted. Any reduction in the number of colonies compared to the air control indicates a bacteriostatic or bactericidal action. The results of a typical experiment with the very effective oxyquinoline vapors and ineffective triethylene glycol after thirty to one hundred and twenty seconds contact are shown in Fig. 3.

The results of testing the dynamic vapors effect on other types of bacteria associated with respiratory infections are shown in Table III.

TABLE III.—ANTIBACTERIAL ACTION OF OXYQUINOLINE VAPORS ON VARIOUS RESPIRATORY BACTERIA

Test Organism	Exposure	Total number of colonies per tube			
		30 sec.	60 sec.	90 sec.	120 sec.
S. aureus	Air control	338	356	356	492
	Oxyquinoline	117	50	0	0
Pneumococcus	Air control	48	52	60	40
Type III	Oxyquinoline	28	1	1	0
Hemolytic	Air control	...[a]	106	126	166
streptococcus	Oxyquinoline	...	26	0	0
H. influenzae	Air control	87	73	97	63
	Oxyquinoline	64	34	50	21

[a] Test not completed.

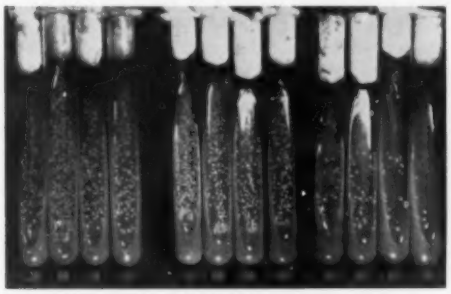

Fig. 3.—Dynamic vapor tests on *S. aureus*. Number of colonies developing in air control (left), triethylene glycol (center), and oxyquinoline (right), after thirty to one hundred twenty seconds exposure to vapors and subsequent incubation for twenty-four hours at 37°. Time of exposure in seconds is shown below each tube.

The results illustrate how rapidly certain vapors inhibit or kill bacteria under the conditions of this test. Of course, to be certain that the absence of growth results from bactericidal rather than bacteriostatic action, subculture tests, similar to those described above, must be carried out.

It is evident that by employing the above described static and dynamic vapor tests for detecting the antibacterial action of volatile substances, it is possible to make a valid quantitative comparison of their bacteriostatic and/or bactericidal action. By means of these tests, many substances have been screened for their potential therapeutic action.

SUMMARY

By means of suitable quantitative experiments it was found possible to screen vapors from volatile compounds for their action on bacteria. It is thought-provoking to observe that a number of vapor-producing compounds which have been used for many years for the inhalation therapy of minor respiratory diseases (thymol, menthol, turpentine, oil of mustard, chlorothymol, etc.) have been shown to liberate vapors which are bactericidal for the types of organisms most frequently associated with respiratory infections, and also display a measurable degree of *in vitro* antiviral action against the influenza virus (17).

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The Identification and Determination of Some Official Local Anesthetics as Tetraphenylborates*

By L. G. CHATTEN, M. PERNAROWSKI, and L. LEVI

The tetraphenylborates of some official local anesthetics may be prepared by interacting the hydrochlorides of the organic bases with sodium tetraphenylboron in aqueous citrate buffers at a pH of 6.0. The reaction affords a high degree of sensitivity, permitting the detection and quantitative estimation of these drugs. The ultraviolet spectra of the derivatives depend, mainly, on the spectral characteristics of the organic bases. Infrared measurements are presented to confirm the mechanism of reaction and to illustrate the value of the IR data for identification purposes.

INTO THAT class of drugs termed local anesthetics are incorporated three general groups of organic compounds: phenols, alcohols, and certain benzoic acid esters of short chain alcohols. Although important, the first two broad groups are overshadowed by the importance of the third group, which includes dibucaine, piperocaine, procaine, and tetracaine hydrochlorides as official drugs in the United States Pharmacopeia as well as naepaine hydrochloride and butethamine hydrochloride which are official in the National Formulary.

Although the official compendia prescribe certain tests and standards for these local anesthetics, new methods for their identification and analyses in pharmaceutical and toxicological materials are continuously being sought (1–5). Several years ago, Wittig and his co-workers found that the tetraphenylboron ion $[B(C_6H_5)_4]^-$ formed compounds with potassium, ammonium, rubidium, and cesium (6, 7, 8), which are very insoluble in water. This observation and subsequent reports that the ion also reacted with basic organic nitrogen compounds led to widespread investigations into the usefulness of the reagent in qualitative and quantitative analyses. Comprehensive literature reviews on the subject have been published by Gloss (9) and Barnard (10, 11, 12).

While the reactions of a wide variety of basic organic nitrogen compounds with sodium tetraphenylboron have been reported in the literature, the local anesthetics have not been extensively investigated. Gautier, Renault, and Pellerin (13) prepared the tetraphenylborates of procaine, tetracaine, and dibucaine. In addition, Schultz and Mayer (14) reported that this reagent would react with certain local anesthetics. Fischer and Karawia (15) reported the preparation of procaine tetraphenylborate and stated the melting range to be 147–151°. Flaschka, Holasek, and Amin (16) showed that the reaction of the tetraphenylboron ion with mercuric chloride resulted in the release of hydrochloric acid. This reaction was used to determine either potassium or various basic organic nitrogen derivatives of tetraphenylboron. Procaine was included in the compounds investigated.

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The authors express their appreciation to Mr. W. Skakum for his assistance with the ultraviolet and infrared spectra, and to Mr. G. Morris for his preparation of the figures.

METHOD

Apparatus.—Beckman pH meter, model G; Beckman glass electrode no. 1190–80; Beckman calomel electrode no. 1170–71; Fisher-Johns melting point apparatus; Cary recording spectrophotometer, model 10; Beckman DU spectrophotometer; Perkin-Elmer infrared spectrophotometer, model 21; and Micro-Kjeldahl apparatus (Scientific Glass Co.).

Reagents and Solutions.—Dibucaine HCl, U. S. P.; piperocaine HCl, U. S. P.; procaine HCl, U. S. P.; tetracaine HCl, U. S. P.; butethamine HCl, N. F.; naepaine HCl, N. F.; methanol AR, obtained from Mallinckrodt Chemical Works; petroleum ether, A. C. S.; acetone, A. C. S.; glacial acetic acid, A. C. S.; sodium tetraphenylboron solution 2% as prepared by Cooper (17); citrate buffers of pH 3, 4, 5, and 6 as described by Clark (18); crystal violet indicator solution prepared by dissolving 500 mg. in 100 ml. of glacial acetic acid; acetous perchloric acid 0.1 *N*; and acetous perchloric acid 0.05 *N*.

PROCEDURES

Establishment of Purity of Local Anesthetics.—Each compound was recrystallized by dissolving 10 Gm. in 25 ml. of ethanol with the aid of heat. The solution was filtered while hot and cooled for thirty minutes in a refrigerator. Crystals were collected on a sintered-glass funnel and dried at 100°. Melting points were determined and nonaqueous titrations carried out potentiometrically in anhydrous methanol using a glass calomel electrode system. The titrant was 0.1 *N* acetous perchloric acid.

Preparation of the Tetraphenylborates of Local Anesthetics.—Add to an accurately weighed sample of about 0.2 Gm. of the local anesthetic or to an accurately measured aliquot of the pharmaceutical product to be analyzed, 20 ml. of citrate buffer (pH 6) and, dropwise, 2% sodium tetraphenylboron solution. Stir the solution well during the addition of the reagent and add approximately 1 ml. excess. Set the mixture aside for about one hour and collect

the precipitate under suction in a sintered-glass crucible of fine porosity. Dry in a vacuum desiccator over phosphorus pentoxide for twenty-four hours.

Volumetric Analysis of Tetraphenylborates.—Dissolve approximately 100 mg. of the local anesthetic tetraphenylborate in 5 ml. of acetone. Add 45 ml. of glacial acid and one drop of crystal violet indicator. Titrate to a blue end point for piperocaine and dibucaine tetraphenylborates and to a green end point for the tetraphenylborates of procaine, naepaine, and tetracaine.

Ultraviolet Measurements.—The spectra of both the hydrochloride salts and the tetraphenylborates were taken in anhydrous methanol. The final dilution was such that concentration, in all instances, ranged from 1 to 2 mg. in 100 ml. of solvent. No heat was used during the preparation of the solutions. Absorbances were determined in 1-cm. cells using either the Beckman DU or Cary recording spectrophotometer. From the data so obtained, the log ϵ values were calculated and plotted against wavelength.

Preparation of Samples for Infrared Examination.—The infrared spectra of the local anesthetics and their tetraphenylborates shown in Fig. 3 were recorded on a Perkin-Elmer Double-Beam Model 21 Spectrophotometer equipped with sodium chloride optics.

The materials were finely powdered in a mechanical grinder until they would pass through a 250-mesh sieve (U. S. Standard Sieve Series No. 230). Accurately weighed amounts were then mixed intimately with sufficient reagent grade potassium bromide A. C. S., treated similarly and dried overnight at 125° to obtain preparations of 0.5% w/w composition. Aliquots of 200 mg. were subjected *in vacuo* to a pressure of 10,000 pounds/sq. inch for about five minutes and the absorbances of the clear disks thus produced measured over the frequency range extending from 4,000–650 $cm.^{-1}$. A potassium bromide disk prepared under comparable conditions was placed in the path of the reference beam to compensate for absorption by the reagent. After each experiment the disks were weighed in order to determine the amount of sample present. The weight variations between different disks never exceeded 1%.

DISCUSSION

The melting points of the purified local anesthetics and the results of the nonaqueous titrations of these compounds are reported in Table I. It is seen from this data that the compounds meet the requirements of purity as established by the "United States Pharmacopeia" (19) and the "National Formulary" (20)

TABLE I.—CRITERION OF PURITY OF LOCAL ANESTHETICS

Compound	Melting Range, °C.	Purity by Non-aqueous Titration, %
Butethamine HCl	193.9–194.6	99.9
Dibucaine HCl	98.8–100.0	100.1
Naepaine HCl	177–178	99.9
Piperocaine HCl	175	100.4
Procaine HCl	156.5–157.6	99.4
Tetracaine HCl	147.5–149.9	98.6

The tetraphenylborates were prepared at pH 3, 4, 5, and 6, in accordance with the procedure described. Because of reports that increased temperatures, under certain conditions, decompose these derivatives (8, 21), the solutions were not heated. It was found that recoveries were essentially the same within a pH range of 3 to 6, provided the sodium tetraphenylboron solution was added slowly and with stirring to prevent co-precipitation of the citrate ions from the buffer. On recovery of the precipitates, there appeared to be little difference in their physical appearance. In general, however, finer precipitates were obtained when the pH of the initial solution was either 5 or 6. On standing, the precipitates, prepared at pH 3 or 4, darkened slightly and developed phenolic-like odors. Only piperocaine tetraphenylborate appeared to be unaffected by pH throughout the range investigated. Percentage recoveries of the tetraphenylborates, prepared at pH 6, are recorded in Table II. Several commercial intravenous pharmaceuticals were also assayed by the proposed procedure and comparative analyses carried out using the official method of the "United States Pharmacopeia" (19). From the data reported in Table III, it is noted that satisfactory agreement was obtained between the two methods.

Using the procedures described in this paper, butacaine tetraphenylborate could not be recovered quantitatively. Excessive clumping resulted in the formation of hard amber lumps and recoveries were erratic. The reaction was studied also with xylocaine, cyclomethycaine, tronethane, larocaine, hexylcaine, panthesin, and diothane. Generally, recoveries of the tetraphenylborates were low, in these instances. No correlation between the chemical structure of the compounds investigated and their recoveries could be detected. Evidently other factors also influence the course of the reaction.

Crane (22) reported that the original tetraphenylborates were quite pure and required no recrystallization. Gautier, Renault, and Pellerin (13) established the purity of the individual derivatives by titrating them in glacial acetic acid with acetous perchloric acid. The indicator was crystal violet. Their technique, with slight modifications, was employed in this laboratory to determine the purity of the tetraphenylborates. From the results of these titrations, as given in table IV, it would appear that the compounds are of such high purity as to obviate the necessity of recrystallization.

Ultraviolet Data.—Results of the ultraviolet studies are shown in Figs. 1 and 2 and summarized in Table V. It was found that, unlike the reagent, the local anesthetics and their tetraphenylborates exhibit marked absorption in the ultraviolet region. It is concluded, therefore, that the absorption of the tetraphenylborate is due primarily to the contribution afforded by the local anesthetic. Thus ϵ maximum values for the hydrochlorides and their tetraphenylborates are in close agreement in every instance. For dibucaine and piperocaine the λ maximum of the hydrochloride and the tetraphenylborate occur at identical wavelengths. However, for procaine, naepaine, tetracaine, and butethamine the λ maximum of the tetraphenylborates are shifted by about 4–5 $m\mu$ toward shorter wavelengths.

The ϵ minimum values of the tetraphenylborates are, in every instance, considerably higher than those

TABLE II.—MELTING RANGES AND PER CENT RECOVERIES OF TETRAPHENYLBORATES OF SOME OFFICIAL LOCAL ANESTHETICS AT pH 6

Procaine		Piperocaine		Naepaine		Tetracaine		Dibucaine		Butethamine	
Melting Range, °C.	Recovery, %	Melting Range, °C.	Recovery, %	Melting Range, °C.	Recovery, %	Melting Range, °C.	Recovery, %	Melting Range, °C.	Recovery, %	Melting Range, °C.	Recovery, %
141–143	99.8	113–116	100.3	110.5–112.5	99.5	119–120	99.2	115.5–118	99.8	91.5–108	Not reproducible
	99.9		99.1		100.7		100.4		100.6		
	99.3		99.2		99.3		99.6		99.1		

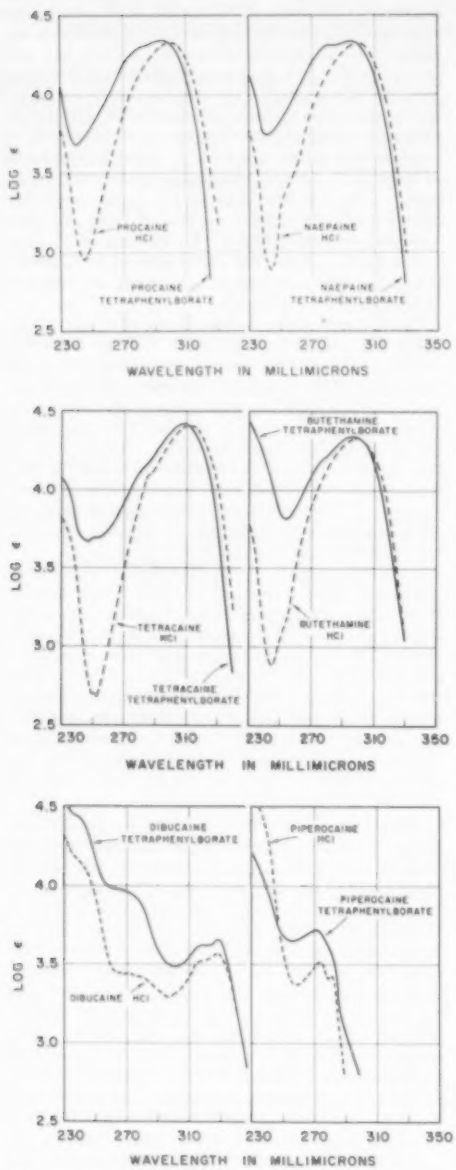


Fig. 1.—Ultraviolet absorption spectra of some local anesthetics and their tetraphenylborates.

TABLE III.—RECOVERY OF PROCAINE FROM INTRAVENOUS PHARMACEUTICALS BY U. S. P. METHOD OF ANALYSIS AND AS TETRAPHENYLBORATE

Manufacturer	Product	Recovery, % Tetraphenylborate	Recovery, % U. S. P.
A	Procaine HCl 2%	103.0	100.4
		102.3	101.9
A	Procaine HCl 2% with epinephrine	99.5	98.3
		99.4	98.6
B	Procaine HCl 2%	101.7	100.4
		101.5	98.3
B	Procaine HCl 2%	98.8	97.6
		98.4	...
C	Procaine HCl 2%	96.0	95.8
		95.1	95.2
C	Procaine HCl 2%	95.4	94.9
		94.2	94.6
D	Procaine HCl 2%	99.6	99.3
		99.2	99.3

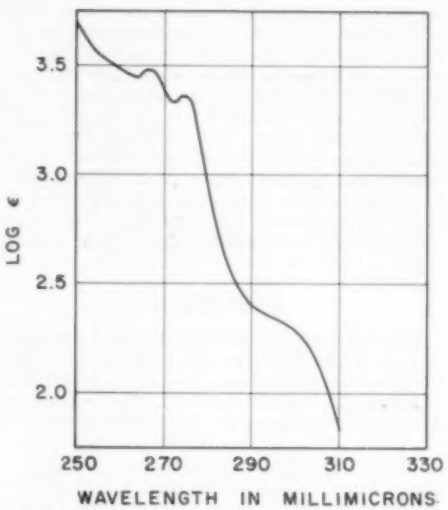


Fig. 2.—Ultraviolet absorption spectrum of sodium tetraphenylboron.

of the corresponding hydrochlorides. The phenomenon is due, obviously, to the effect exerted by the tetraphenylboron moiety in the molecule. In no instance does the λ minimum of the tetraphenylborate coincide with that of the hydrochloride. In some instances, the former occurs at a slightly higher wavelength and in others at a slightly lower one.

As one would anticipate from the similarity of

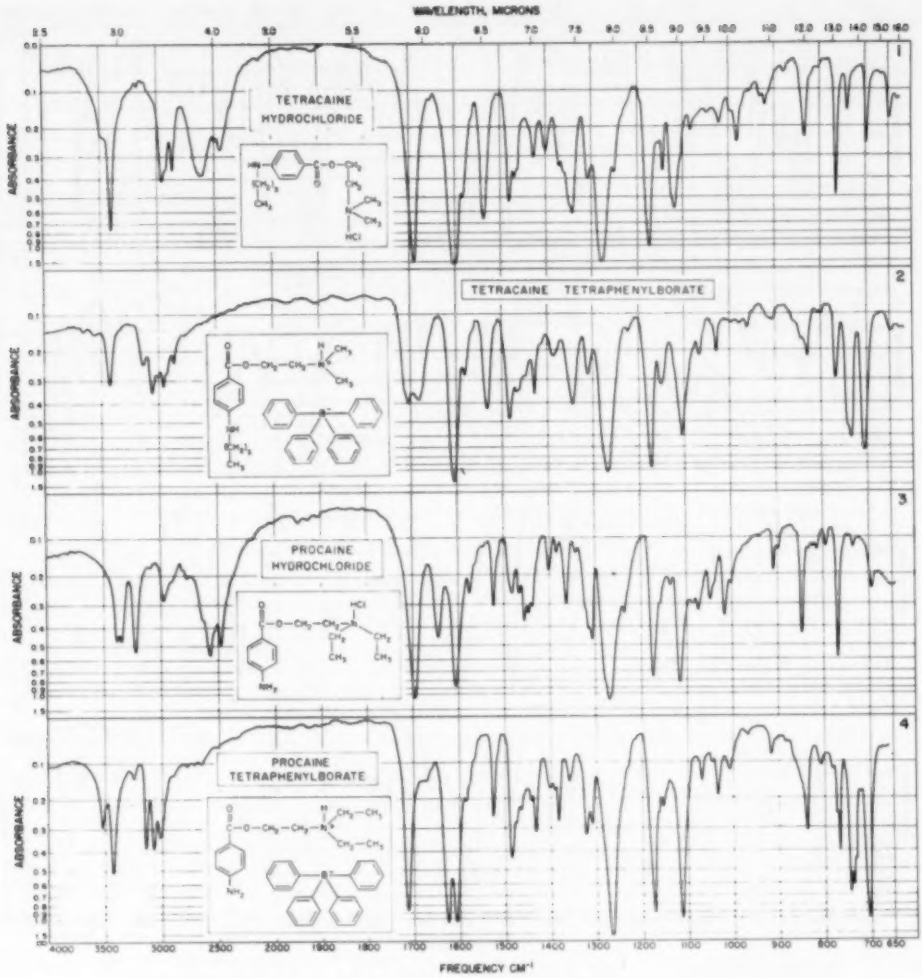


Fig. 3.—Infrared spectra of some local anesthetics and their tetraphenylborates.

their structural formulas, the spectra of procaine, naepaine, and tetracaine are remarkably alike. The λ maximum of the first two compounds occur so closely together, at 299 to 300 mμ, that it is impossible to differentiate between them. Tetracaine can, on the other hand, be distinguished by the fact that its maximum occurs at 314 mμ. The λ minimum of butethamine tetraphenylborate is of value in distinguishing it from procaine and naepaine. For butethamine, it occurs at 253 mμ whereas the λ minimum of the latter two compounds is observed at 240 and 241 mμ, respectively. With this exception, the tetraphenylborate spectra appear to have no advantage over those of the hydrochlorides. It appears that none of the spectra will permit differentiation of procaine and naepaine.

Both dibucaine and piperocaine can be differentiated readily from each other and from the remainder of the local anesthetics considered in this report by either their hydrochloride or tetraphenylborate spectra. This is to be expected however, when one considers the differences in their chemical structure. Dibucaine contains a quinoline group and piperocaine the piperidine moiety.

Infrared Data.—The infrared spectra of the compounds are shown in Fig. 3. The spectrum of sodium tetraphenylborate (Fig. 4) displays two intense bands in the 700–750 cm.$^{-1}$ region (out-of plane C-H bending vibrations of the phenyl groups) and in the C = C stretching region two marked absorptions are observed in the 1,500 and 1,600 cm.$^{-1}$ wavelength range, respectively (C = C skeletal in-plane vibrations). Characteristic = C-H stretching vibrations occur in the form of a doublet throughout the 3,000-cm.$^{-1}$ region.

Comparison of the spectra of the local anesthetics with those of the tetraphenylborates shows that the reaction proceeds in accordance with the equation:

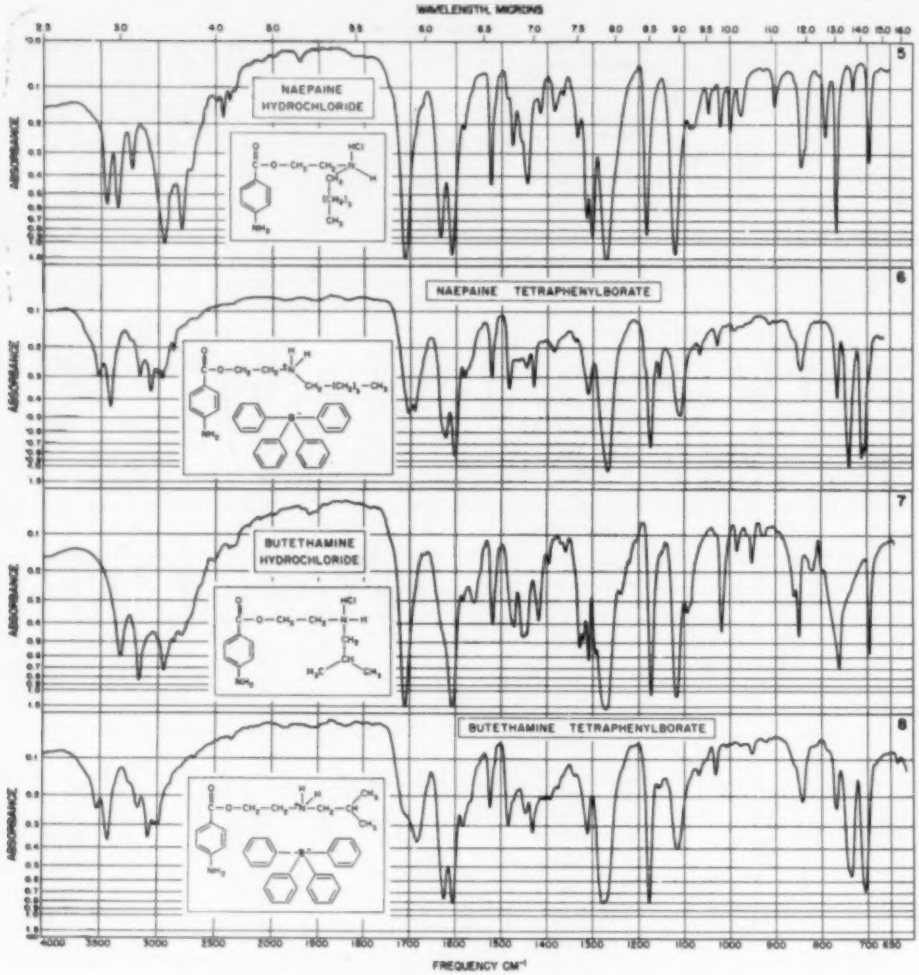
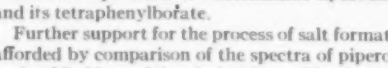


Fig. 3.—Infrared spectra of some local anesthetics and their tetraphenylborates.

$$[RN{:}H]^+[Cl]^- + [Na]^+[(C_6H_5)_4{:}B]^- \rightarrow [RN{:}H]^+[(C_6H_5)_4{:}B]^- + NaCl$$

The mineral acid salts of the organic bases generally display absorptions near 2,500 cm.$^{-1}$ (N-H stretching vibrations observed in amine hydrochlorides) (23). Their derivatives, on the other hand, exhibit no structure in this region which observation is in accord with the reaction mechanism illustrated in the equation shown. The phenomenon is most pronounced in the spectra of tetracaine and procaine hydrochloride and their derivatives and least noticeable in the spectrum of butethamine hydrochloride and its tetraphenylborate.

Further support for the process of salt formation is afforded by comparison of the spectra of piperocaine hydrochloride and its derivative. The local anesthetic shows no structure in the 3,500–3,600 cm.$^{-1}$ region, whereas a characteristic doublet in this wavelength range is observed in the spectrum of the tetraphenylborate. The absorption is to be ascribed to oscillations of the type of N-H bond formed as a result of the reaction which establishes, generally, an electrostatic field of attraction between the organic bases and the tetraphenylboron moiety as shown in the structural formulas. Infrared evidence thus illustrates how the vibration characteristics of the N-H linkage present in the reactant are modified because of environmental effects in the derivative. The phenomenon is not clearly recognizable in the spectra of the remaining tetraphenylborates included in this study because of the presence of both



N-HCl and N-H groupings in the local anesthetics prior to reaction. It should be noted in this connection that the absence of normal N-H stretching bands in the chloroform spectra of piperidine and

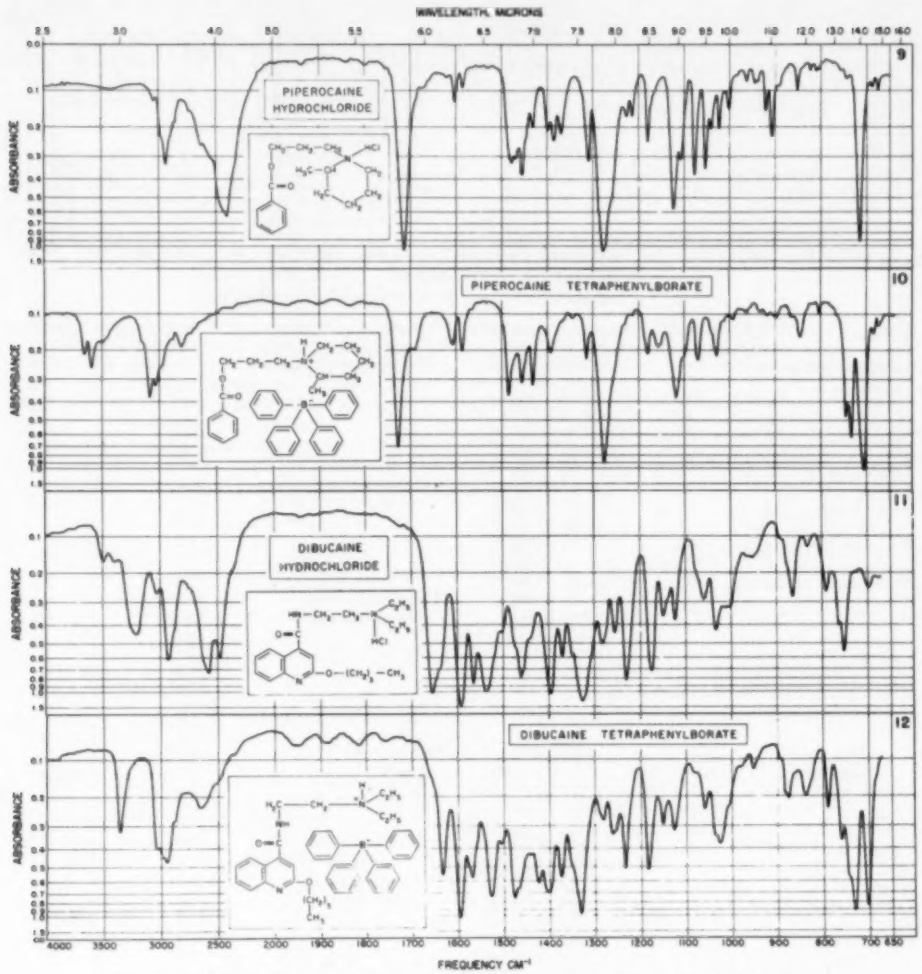


Fig. 3.—Infrared spectra of some local anesthetics and their tetraphenylborates.

some natural products containing this ring system was observed previously by Marion, Ramsay, and Jones (24). The phenomenon evidently also applies to the synthetic local anesthetic piperocaine (benzoic ester of γ-(2-methyl piperidino)-propanol) but is absent in the tetraphenylboron derivative.

The local anesthetics studied display several common characteristic structural features and, since these features are retained in the reaction product, the two series of spectra exhibit a number of common bands.

Characteristic N-H and C-H stretching absorptions are observed in the 3,500 and 3,000 cm.$^{-1}$ region, respectively. Strong bands also occur in the 1,700 cm.$^{-1}$ region because of the presence of a carbonyl group in these molecules. The absorption is both modified and displaced as a result of derivatization. In dibucaine hydrochloride it appears at 1,654 cm.$^{-1}$, which position is normal for secondary amines examined in the solid state. In the tetraphenylborate it is observed at 1,635 cm.$^{-1}$. In the spectrum of butethamine hydrochloride it appears as an intense, sharp band at 1,715 cm.$^{-1}$, whereas in the tetraphenylborate compound it is broadened and displaced toward lower frequency at 1,690 cm.$^{-1}$. In both procaine and piperocaine hydrochloride the band is intense, occurring at 1,700 and 1,718 cm.$^{-1}$, respectively. Their tetraphenylborates also display the absorption as a sharp peak but at shorter wavelengths (1,718 and 1,731 cm.$^{-1}$, respectively). In the spectra of tetracaine and naepaine tetraphenylborate the band is split into a doublet. Since identical concentrations were used for the measurements the absorption is invariably more intense in the spectra of the local anesthetics than in the corresponding derivatives.

Intense absorption as either a single band or a doublet is observed in the 1,600 cm.$^{-1}$ region. It

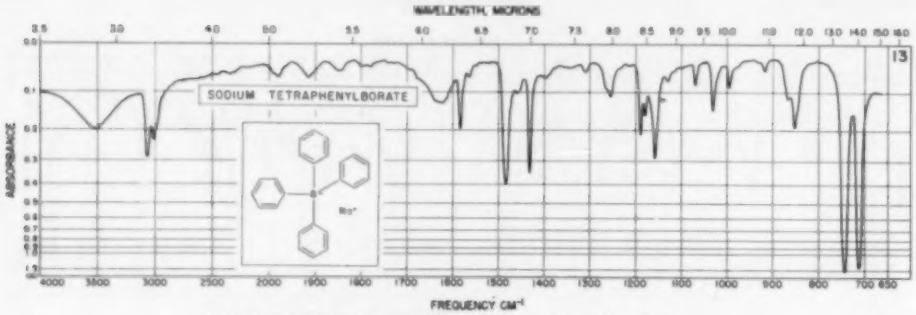


Fig. 4.—Infrared spectrum of sodium tetraphenylboron.

TABLE IV.—TITRATION OF TETRAPHENYLBORATES WITH PERCHLORIC ACID

Tetraphenylborate	Equivalent	Taken, mg.	Recovered, mg.	Purity, %
Naepaine	M/2	101.6	100.1	98.5
		102.9	102.0	99.1
Dibucaine	M/2	102.2	101.2	99.0
		110.0	109.7	99.7
Piperocaine	M	130.9	131.1	100.2
		116.2	116.0	99.8
Procaine	M/2	101.0	100.0	99.0
		105.0	104.5	99.5
Tetracaine	M/2	101.3	100.9	99.6
		102.2	101.7	99.5

TABLE V.—ULTRAVIOLET CHARACTERISTICS OF SOME LOCAL ANESTHETICS AND THEIR TETRAPHENYLBORATES

Compound	λ Maximum, mμ	ε Max.	λ Minimum, mμ	ε Min.
Procaine HCl	300	21680	245	885
Procaine T.P.B.	295	22315	240	4841
Piperocaine HCl	274	3300	259	2365
Piperocaine T.P.B.	274	5261	256.5	4485
Naepaine HCl	299	20930	244	794
Naepaine T.P.B.	295	22178	241	5576
Tetracaine HCl	314	25627	252	482
Tetracaine T.P.B.	309	26244	246.5	4742
Dibucaine HCl	328	3760	297	1960
Dibucaine T.P.B.	328	4527	300	3160
Butethamine HCl	299	21271	245	779
Butethamine T.P.B.	295	22520	253	6672
Sodium T.P.B.	266	3002	264	2853
	274	2271	272	2122

may be considered indicative of the deformation frequencies of the C_6H_5-N bonds occurring in these molecules although its exact interpretation is complicated by the fact that aromatic ring vibrations also occur in this wavelength range. Piperocaine hydrochloride and its derivative, which compounds lack the anilino structure, do not show this absorption.

Further intense bands are observed in the 1,275-, 1,175-, and 1,125-cm.$^{-1}$ regions (C-N stretching vibrations of aromatic amines and C-O stretching vibration of aromatic esters). Only dibucaine hydrochloride, which is not an ester but an amide contain-

TABLE VI.—POSITION OF KEY BANDS IN INFRARED SPECTRA OF LOCAL ANESTHETICS AND THEIR TETRAPHENYLBORATES IN THE 850–650 CM.$^{-1}$ REGION

Compound	Characteristic Vibration Frequency, cm.$^{-1}$	Intensity of Absorption
Tetracaine hydrochloride	834	s
	810	i
	765	vs
	740	m
	698	s
	648	m
Tetracaine tetraphenylborate	831	m
	810, 803	Doublet
	770	s
	745	sh
	736	s
	708	s
	650	w
Procaine hydrochloride	848	vs
	835–815	Triplet
	795	w
	770	vs
	736	w
	695	w
Procaine tetraphenylborate	840	s
	806	w
	784	vw
	773	w
	769	s
	745, 738	Doublet
	733	w
	705	vs
Naepaine hydrochloride	845	s
	837	vw
	792	s
	768	vs
	732	w
	695	vs
Naepaine tetraphenylborate	847	m
	767	s
	740	s
	712, 706	Doublet
Butethamine hydrochloride	850	s
	825	w
	800	i
	763	vs
	697	vs
Butethamine tetraphenylborate	845	m
	787	i
	770	m

TABLE VI. (*cont.*)

Compound	Characteristic Vibration Frequency, cm.$^{-1}$	Intensity of Absorption
Butethamine tetraphenylborate (cont.)	743	i
	737	s
	708	vs
	635	vw
Piperocaine hydrochloride	850	w
	823	vw
	810	vw
	805	vw
	745	vw
	715	vs
	688	vw
	675	vw
Piperocaine tetraphenylborate	847	w
	805	vw
	743	Doublet
	735	
	707	vs
	685	vw
	675	vw
Dibucaine hydrochloride	833	w
	792	w
	763	vw
	753	s
	700	w
Dibucaine tetraphenylborate	840	m
	790	s
	762	w
	745	i
	732	vs
	703	vs

ing a quinoline structure, displays a different absorption pattern. The presence of the heterocyclic ring system is recognized by the four bands (two strong and two weak) observed in the 1,600–1,500-cm.$^{-1}$ region (C = C and C = N stretching vibrations) and by the absorptions occurring at 865 and 736 cm.$^{-1}$ (C – H deformation vibrations). In the tetraphenylborate derivative the 1,600–1,500-cm.$^{-1}$ profile remains unaltered but in the fingerprint region, which is more sensitive to total molecular structure, the absorptions are modified.

In addition to the spectral features which reflect the presence of specific functional groups in both the parent compounds and their derivatives there are also found a number of characteristic bands which may be used to differentiate the various molecules from one another. Thus the reaction is of value to the forensic chemist and toxicologist. In general, the spectra of the tetraphenylborates are richer in structure than those of the local anesthetics, particularly throughout the 850-650 cm.$^{-1}$ region where spectral contributions of the reagent are found to be maximal. This wavelength range is therefore most useful for identification purposes, as shown in Table VI which lists the characteristic absorptions occurring in this region.

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In Vitro Test for Antiarrhythmic Agents*

By CHESTER W. DURACHTA†, and HUGH C. FERGUSON‡

A valid method for screening antiarrhythmic agents is described. Methods of producing and recording various types of arrhythmias are outlined, and response to standard drugs discussed.

THE MASS SCREENING of potentially useful medicinal agents should be rapid, reliable, and inexpensive. Preliminary evaluation of a new substance should indicate whether additional experiments might be required or that further studies would be fruitless. The initial assay should be simple, calculations and technical manipulations kept at a minimum, and the results clearly defined and unequivocal. If these conditions are satisfied the time saved could be used for additional screening, or active compounds could be subjected to more critical examination with the ultimate objective of clinical utility.

Methods for the production of auricular arrhythmias and ventricular fibrillation in the isolated rabbit heart have been described by Ferguson and Durachta (1). It was thought that this preparation might be used as a rapid screening

* Received October 15, 1958, from the Division of Biological Research, G. D. Searle and Co., Chicago, Ill.
† Present Address: Smith Kline and French Laboratories, Philadelphia, Pa.
‡ Present Address: Ohio Northern University, College of Pharmacy, Ada.

method if the experimentally induced arrhythmias would respond favorably to clinically efficacious compounds such as quinidine sulfate, procainamide, and methacholine chloride. Therefore, studies were undertaken in order to determine the effects of these agents on the arrhythmias so produced.

EXPERIMENTAL

Methods.—Rabbits weighing 2.5–3.0 Kg. were sacrificed by a sharp blow on the head. The hearts were rapidly extirpated and set up as originally described by Langendorff (2) and modified by Locke and Rosenheim (3). Auricular flutters or paroxysmal tachycardias were induced electrically by stimulating the right auricle with 5–10 volts, square waves, at a pulse duration of 5–10 milliseconds, with a pulse delay of 5–10 milliseconds and a frequency of 50–150 cycles/second. If persistent auricular irregularities could not be instituted after ten to fifteen minutes, ventricular fibrillation was initiated by applying shocks of 100–140 volts, with no pulse delay, alternately and rapidly to both atria with a Grass stimulator. The latter technique was always immediately successful and spontaneous reversions of fibrillation were never observed. Auricular arrhythmias could also be produced by the topical application of 0.05% solution of aconitine nitrate. All compounds to be tested were prepared in Locke-Ringer's solution at a concentration of 200 μg./0.1 cc. Compounds were administered into the perfusate via a side arm cannula at geometrically progressive doses starting at 200 μg. Electrical potentials were recorded from lead II (right auricle-left ventricle) by means of a Twin Viso Sanborn Electrocardiograph (calibration: 1 millivolt = 5 or 10 mm., paper speed = 25 mm./sec.) at one, three, and five minutes after administering the compound to be tested, and if the arrhythmia persisted, the next higher dose was administered. This dosage schedule was continued until reversion to a normal sinus rhythm occurred or until a definite effect was produced.

RESULTS

In 49 experiments, persistent auricular flutters were initiated 22.5% of the time, paroxysmal tachycardias were obtained in 53.1% of the experiments, and ventricular fibrillations could always be produced. The results are summarized in Table I. When auricular arrhythmias could not be instituted compounds were screened for their effect against ventricular fibrillation.

TABLE I.—RESULTS OF EFFORTS TO PRODUCE PERSISTENT ARRHYTHMIAS IN THE ISOLATED RABBIT HEART

Arrhythmia Produced	Number of Trials	Number Successful	Successful, %
Atrial flutter	49	11	22.5
Auricular paroxysmal tachycardia	49	26	53.1
Ventricular fibrillation	21	21	100.0

Quinidine Sulfate.—This compound was tried against auricular paroxysmal tachycardia, auricular flutter, and ventricular fibrillation and was active at an average dose of 0.431 ± 0.038 mg. (Table II). Figure 1 shows typical electrocardiographic potentials obtained before and during fibrillation and three minutes after the administration of 0.500 mg. of quinidine sulfate, at which time reversion to a normal sinus rhythm occurred. In all experiments, reversions to normal sinus rhythm were seen one to five minutes after administration of this agent.

Procainamide (Pronestyl).—This substance was tried on experimentally induced ventricular fibrillation and was active at an average dose of 1.58 ± 0.135 mg. (Table II). In all experiments reversions to normal sinus rhythm occurred one to five minutes after compound administration. Typical potentials seen during ventricular fibrillation are demonstrated by the electrograms in Fig. 2. In this experiment reversions to a normal sinus rhythm occurred approximately three minutes after administration of 1.6 mg. of procainamide.

Methacholine Chloride.—Mecholyl was active against auricular paroxysmal tachycardia at an average dose of 0.0043 mg. ± 0.0003 mg. (Table II), but was ineffective against atrial flutter and ventricular fibrillation. Auricular paroxysmal tachycardia is demonstrated by electrocardiographic potentials shown in Fig. 3. This irregularity was reverted to normal sinus rhythm one to two minutes

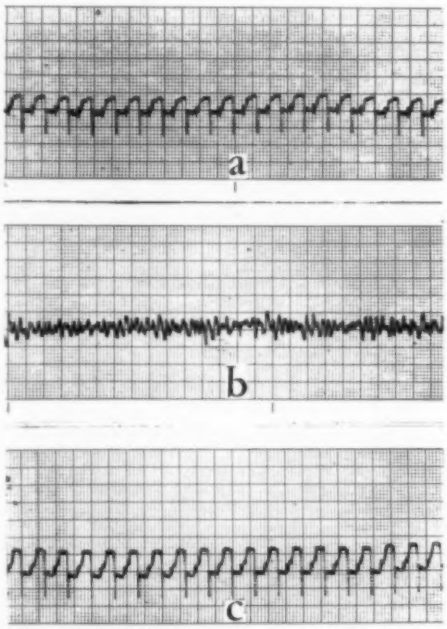

Fig. 1.—Electrocardiographic potentials obtained from the isolated rabbit heart. Calibration 1 mv. = 10 mm., paper speed = 25 mm./sec., lead II (= right auricle-left ventricle). (a) Pretreat. record. Heart rate = 225 beats/minute. (b) Electrically induced fibrillation. (c) Record three minutes after administration of 0.500 mg. quinidine sulfate. Heart rate = 225 beats/minute.

TABLE II.—EFFECT OF VARIOUS ANTIARRHYTHMIC AGENTS ON ARRHYTHMIAS IN THE ISOLATED RABBIT HEART

	Quinidine Sulfate	Procainamide	Methacholine Chloride
Type(s) of irregularity induced	Auricular flutter Ventricular fibrillation Paroxysmal tachycardia	Fibrillation	Paroxysmal tachycardia
Number of trials	19	16	11
Successful reversions	19	16	11
Active dose (mg.)	$\bar{X} = 0.431$ ± 0.038	$\bar{X} = 1.580$ ± 0.135	$\bar{X} = 0.0043$ ± 0.0003

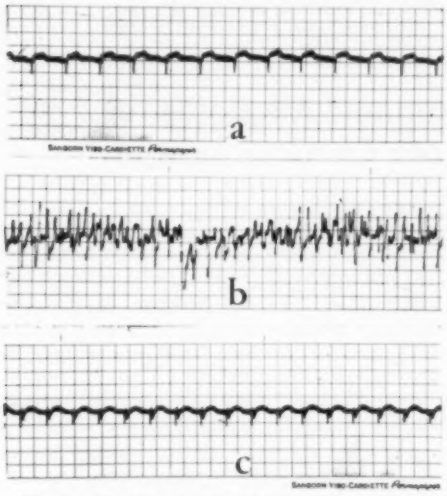

Fig. 2.—Electrocardiographic potentials obtained from the isolated rabbit heart. Calibration 1 mv. = 10 mm., paper speed = 25 mm./sec., lead II (= right auricle-left ventricle). (a) Pretreat. record. Heart rate = 90 beats/minute. (b) Electrically induced fibrillation. (c) Record three minutes after administration of 1.6 mg. procainamide. Heart rate = 120 beats/minute.

after the administration of 0.001 mg. of methacholine chloride.

In conditions other than auricular paroxysmal tachycardia, bradycardia or complete arrest occurred following larger doses of methacholine chloride; upon resumption of cardiac activity no remission of auricular flutter or ventricular fibrillation was seen.

DISCUSSION

Our results demonstrate that the Langendorff heart can be easily modified for use as a screening tool for antiarrhythmic agents. Clinically efficacious compounds such as quinidine sulfate, procainamide (Pronestyl), and methacholine chloride have produced remissions of cardiac irregularities in the isolated rabbit heart which are similar to those found in human cardiac patients. The quantity of material needed is very small and this lends itself well to screening programs where newly synthesized compounds are not generally in great supply. Unfortunately, water insoluble compounds cannot reliably be tested by this method and must be tried on intact preparations as those employed by Rosenbleuth and Garcia Ramos (4, 5), Scherf (6), and Harris (7). It has been shown that, while auricular flutter and

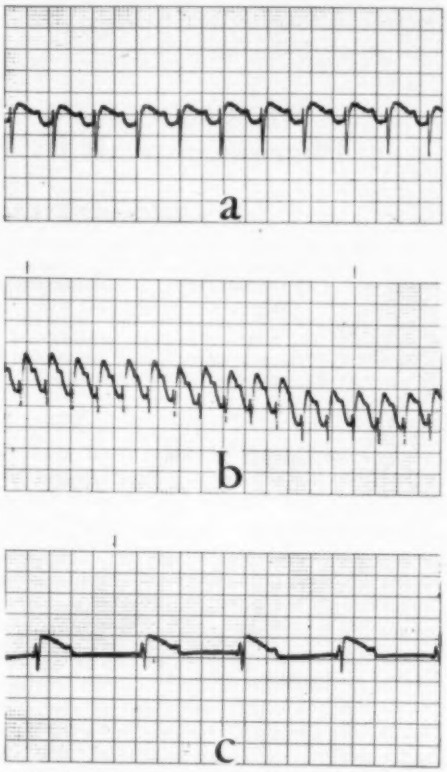

Fig. 3.—Electrocardiographic potentials obtained from the isolated rabbit heart. Calibration 1 mv. = 10 mm., paper speed = 25 mm./sec., lead II (= right auricle-left ventricle). (a) Pretreat. record. Heart rate = 142 beats/minute. (b) Electrically induced paroxysmal tachycardia. (c) Record two minutes after administration of 0.1 ml. of 1:100,000 methacholine chloride (0.001 mg.). Heart rate = 48 beats/minute.

paroxysmal tachycardia cannot always be instituted the preparation need not be discarded since ventricular fibrillation can always be initiated by appropriate electrical stimulation. Determination of the average active dose of standard compounds such as quinidine sulfate, procainamide, or methacholine chloride reveals the relative potency of unknown substances, and determination of the LD_{50} permits an estimate of a compound's margin of safety. Ultimately, knowledge of these factors assists in choosing the safest and most active agents.

SUMMARY

Methods for producing various types of arrhythmia in the isolated rabbit heart have previously been presented. These methods were thought to be potentially useful in screening for antiarrhythmic agents if the irregularities would respond favorably to clinically efficacious agents. Quinidine sulfate, procainamide, and methacholine chloride were tried and found to be consistently active at low doses. It has been our experience that this is a rapid, reliable, and inexpensive method for screening antiarrhythmic agents.

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Microestimation of Opium Alkaloids in Pharmaceuticals by Paper Chromatography*

By KLAUS GENEST and CHARLES G. FARMILO

A method of analysis for morphine, codeine, thebaine, and papaverine in mixtures employing paper chromatographic separation followed by densitometric evaluation with a self-integrating densitometer has been developed. The method has been applied to a number of pharmaceuticals and the results compared with those of other methods.

THE NEED for improvement of existing methods for identification and quantation of opium alkaloids was pointed out in a recent United Nations report (1a). Genest and Farmilo (1b) recently reviewed paper chromatographic analysis of narcotics and found only a few publications which deal with quantitative analysis of opiates. Svendsen (2) estimated morphine in opium by means of a colorimetric method after elution from the chromatogram. Svendsen, Aarnes, and Paulsen (3) compared this method with a densitometric method in which the density of the morphine spot (Folin-Ciocalteu reagent) is measured directly on the paper. Asahina and Ono (4–7) estimated five principal opium alkaloids directly on the paper chromatogram by measuring the maximum absorbance in the U. V. region. Schultz and Strauss (8) applied manual planimetry of chromatograms stained with a ferrichloride-ferricyanide reagent for the estimation of morphine in opium tablets. Paerregaard (9) determined morphine in urine by polarography of the morphine eluted from the chromatogram. Miram and Pfeifer (10) reported a chromatographic method for the analysis of pure solutions of codeine and papaverine requiring the production of a contact negative copy of the chromatogram which was subsequently scanned with the photometer. This step was followed by planimetry of the densitometric trace of the copy to obtain standard area *vs.* concentration graphs. Holubek, Kudrnáč, and Novák (11) estimated codeine and narcotine in poppy capsules after paper chromatographic separation by colorimetry and polarography, respectively.

It is the object of this publication to describe a quantitative paper chromatographic method for opiate analysis which avoids elution, photographic reproduction, or manual planimetry by use of a self-integrating densitometer.

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EXPERIMENTAL

Apparatus and Materials.—The chromatography was conducted by a descending technique on Whatman No. 1 paper strips, one inch wide, using cylindrical glass tanks (12 in. x 24 in.). Ten strips were run in each tank at one time. Details on the salting of the paper with $(NH_4)_2SO_4$ (2%), the preparation of the solvent (iso-BuOH:AcOH:H_2O-10:1:2.4) and the spray-reagent (potassium iodoplatinate) and other chromatographic conditions are the same as those that have been described previously (12, 13), for an ascending technique.

The alkaloid solutions to be estimated were spotted by means of an "Agla"-micrometer syringe. Five λ of a test solution were applied per strip yielding a spot not larger than 5 mm. in diameter. The distance from the solvent to the origin of the chromatogram was 8 cm.

The Spinco "Analytrol" Model RA[1] with focused tungsten lamp, $1^1/_2$ mm. aperture and B-2 cam (absorbance) was used. No filter was employed in the final procedure. The time for scanning of a chromatogram of 40 cm. length containing four spots was approximately three minutes.

PROCEDURE

Preparation of Standard Curve.—Standard solutions of each of the four alkaloids; morphine, co-

[1] See Beckman/Spinco Bulletin RIM-5 for description and operation of "Analytrol."

deine, thebaine, and papaverine, containing from 1 to 10γ/λ in methanol, were prepared. After spotting 5λ of this solution on the strips they were equilibrated with the aqueous phase of the solvent for six hours, after which time the mobile phase was poured into the jar through a separatory funnel. Development required sixteen hours (overnight), in which time the solvent front traveled about 38 cm. After drying, the chromatograms were thoroughly sprayed on both sides with the potassium iodoplatinate reagent and dried again for fifteen minutes in a current of air prior to scanning in the densitometer. The densitometer was calibrated each time another spot was measured. This was done by means of a neutral density filter using the area of the strip containing only chromogenic agent immediately in front of each stained spot. Twenty to thirty strips (in batches of five each) were chromatographed for each concentration of each alkaloid. Standard curves prepared from these data can be seen in Fig. 1.

Application to Pharmaceuticals.—One practical application of the method is demonstrated by using nine pharmaceuticals containing one or more opium alkaloids. The extraction of tablets was made by shaking the powdered material mechanically for half an hour with water. In case of free bases, or slightly soluble alkaloidal salts the extraction was made with glacial acetic acid and diluted with water to a final concentration of 50% acetic acid. The extract was centrifuged and an aliquot of liquid containing between 10 and 30γ of the alkaloid was spotted.

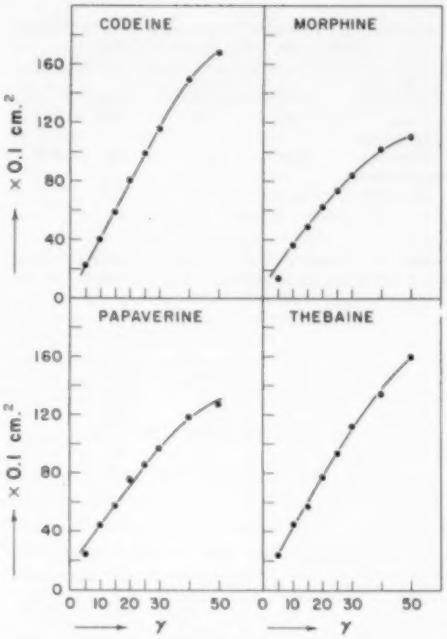

Fig. 1.—Standard curves of four opium alkaloids.

RESULTS AND DISCUSSIONS

Background Color.—The background color of the chromatograms presents a critical problem influencing the simplicity and reproducibility of this method. Considerable investigation of the details of the effect of the variables controlling this color was made. Salting of the papers with phosphates, citrates, or acetates gave brown to pink background colors after spraying. These colors gradually fade to a yellowish shade. The light beam of the densitometer could not penetrate these chromatograms without the use of agents which improve transparency (glycerol, bromo-naphthalene in paraffin oil, varnish, etc.). Salting of the paper with $(NH_4)_2SO_4$ proved to be preferable since it fulfills a dual purpose: round spots without trailing and good separations are obtained, and a background of slightly gray to pinkish color is produced which is easily penetratable by the light ray. Although this combination of salting and chromogenic agent is the choice condition for quantation there are small differences in absolute background density of different chromatograms. To examine the influence of these changes of the absolute density of the background on the reproducibility of the results, artificial backgrounds were created in the following way: A spot of methyl violet on a Whatman No. 1 strip was scanned both with and without sheets of transparent paper between the background of the strip and the scanning photocell. The absorbance (expressed in mm.) of the combined chromatogram plus transparent paper was then recorded. The densitometer chart recording pen was then reset to the zero line, thereby suppressing the enhanced background and the spot was scanned again. The results shown in Table I indicate that suppression of the background up to 19 mm. has little influence on the total absorbance of the methyl violet spot. In replicate chromatograms, the difference in background readings was within ±5 mm.

Cams and Filters.—Experiments to demonstrate the effect of scanning the same chromatograms with different cams and filters were carried out. The results can be seen in Table II. The difference in shade of the spots of the four alkaloids becomes evident by the dissimilar relative response when scanned with and without the filter. Scanning with a blue filter gives a higher total absorbance than is obtained without the filter. However, the irregularities of the background color combined with the texture of the paper were too marked to allow a proper baseline adjustment close to the zero line which militated against its use. Irregularities of background were still more evident when using the densitometer B-1 cam along with other filters. The absorbances were mostly out of range of the instrument for the 35γ level and were not reproducible. Densitometer cam B-2 without a filter was therefore chosen as the equipment for the quantation of opiate chromatograms.

Machine Error.—A spot of red ink on a Whatman No. 1 strip and a morphine spot on a chromatogram were scanned ten times each to estimate the error of replicate scanning. The standard deviations were found to be ±1.45% and ±0.75%, respectively.

Stability of Color.—The alkaloid iodoplatinate stained spots of the four opiates were quite stable after spraying for periods from fifteen minutes to six

TABLE I.—MEASUREMENT OF CHANGES IN BACKGROUND DENSITY BY ANALYTROL

Background Adjustment	Total Color Density (Area Under Curve in 0.1 cm.²)			Average
Normal	135	137	136	136.0
Suppression, 6.5 mm.	138	135	136	136.4
Suppression, 19.0 mm.	138	137	138	137.7

TABLE II.—SCANNING OF A PAPER CHROMATOGRAM WITH DIFFERENT CAMS AND WITH AND WITHOUT BLUE FILTER[a]

Alkaloid[b]	Cam No. B₂, no filter	B₂, filter	B₃, no filter	B₃ filter
Papaverine	64	100	29	51
Thebaine	97	128	50	74
Codeine	108	122	66	82
Morphine	83	83	50	48

[a] Corning 5031.
[b] 30γ of each alkaloid.

hours. Results of the color stability study are given in Table III. If chromatograms cannot be scanned immediately after drying (15 min.) care should be taken to protect them from daylight. Three strips stored in the dark were rescanned after three months and showed an average decrease in absorbance for papaverine, thebaine, codeine, and morphine of 9, 7, 3, and 2%, respectively.

Standard Curves.—Figure 1 shows the standard curves of four opiates. The plotted points represent the average of 20 estimations for each concentration of alkaloid. The experiments to obtain these results were made on consecutive days at room temperature (26° ± 1°), in one chromatographic tank using the same lot of salted paper for each experiment. The average of the standard deviations of 640 experiments was ±3.94%. The standard curves show a linear concentration *vs.* total density relationship from 5γ to approximately 30–40γ. The best reproducibility was found between 10 and 30γ. Figure 2 shows a reproduction of an original chromatogram including the densitometer trace. The jagged lines between the spots

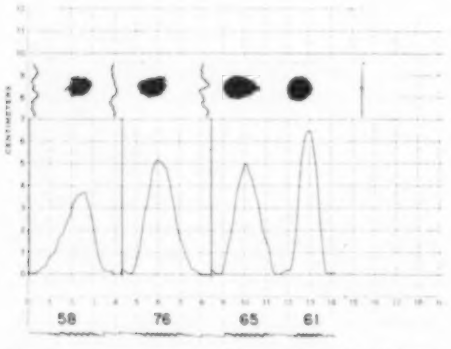

Fig. 2.—Chromatogram of four opium alkaloids and evaluation by "Analytrol."

TABLE III.—STABILITY OF COLOR

Time, min.	Total Color Density (Area Under Curve in 0.1 cm.²) Papaverine[a]	Thebaine[b]	Codeine[a]	Morphine[a]
15	71	92	110	60
30	71	91	104	60
40	66	87	102	59
55	66	88	101	61
65	67	88	102	58
120	68	92	104	59
240	66	87	100	60
360	63	87	103	60
Next morning (22 hr.)	64	84	101	62

[a] 20γ. [b] 25γ.

in Fig. 2 indicate where the original chromatogram was cut for mounting above the maximum of the densitometer trace. (R_f values for morphine, codeine, thebaine, and papaverine on this chromatogram were found to be 0.09, 0.18, 0.56, and 0.72, respectively.) Mounted below the spots in Fig. 2 are the bell-shaped tracings of absorbance of the alkaloidal spots. The serrated line, at the bottom of Fig. 2 represents the tracing of the densitometer integrating pen. The area under the curve corresponds to the total color density since the B-2 cam is calibrated in terms of absorbance. One tooth of the serrated trace corresponds to 0.1 cm.².

Application to Pharmaceuticals.—The paper chromatographic method was applied to a variety of pharmaceuticals which contained, in addition to opium alkaloids, a number of other active ingredients as shown in Table IV. All analytical values are averages of duplicates. The results in Table IV indicate agreement between the values obtained by the chromatographic method and the methods of comparative assay.

The sets of experiments to reproduce the standard curves were repeated at intervals. It was found that the calibration graphs were sometimes not reproducible within the standard deviation

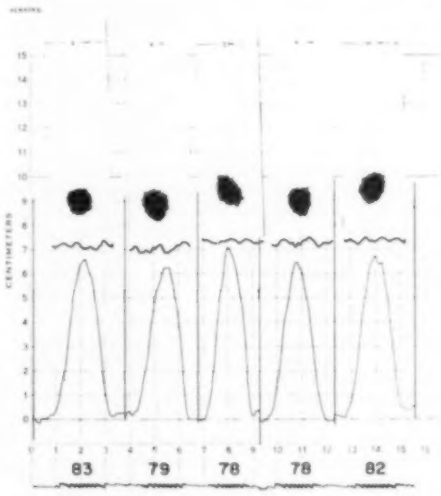

Fig. 3.—Replicate analysis of APC and C tablets.

TABLE IV.—PAPER CHROMATOGRAPHIC AND COMPARATIVE ANALYSIS OF PHARMACEUTICALS

Drug	Opium Alkaloids	Other Active Ingredients	Medium of Extraction	Labeled Strength, mg.	Densitometric Assay, mg. By Standard Curve, mg.	Densitometric Assay, mg. By Comparison,[a] mg.	Comparative Assay, mg.	Method for Comparative Assay
A	Papaverine	Nicotinic acid, nicotinamide	Acetic acid	80	80	...	80.7	$CHCl_3$-Shake out, Reineckate (14)
B	Papaverine·HCl	None	Acetic acid	15	15.3	14.9	14.9	UN-K/34 (15)
	codeine phosphate			15.	15.7	16.4	15.2	
C	Morphine HCl	Tinct. chloroformi et morphinae co.	None (dilution with ethanol)	(1%)	(0.96%) (1.07%)	... (1.06%)	(0.99%)	BPC 1954
D	Codeine phosphate	APC[b]	H_2O	32	32.6	31.6	32.4	Nonaqueous titration (16, 17)
E	Codeine phosphate	APCC[c]	H_2O	8	8.7	8.9	8.4	Nonaqueous titration (16, 17)
F	Codeine phosphate	APCC[c]	H_2O	16	15.3	15.3	16.3	Nonaqueous titration (16, 17)
G	Codeine phosphate	APCC[c]	H_2O	32	30.8	30.6	31.7	Nonaqueous titration (16, 17)
			acetic	32	33.3	34.7	...	
H	Morphine sulphate	None	H_2O	16.2	18.3	16.9	17.0	BP (1948)
I	Morphine sulphate	None	H_2O	10.8	11.4	11.4	11.1	BP (1948)

[a] Value obtained by comparison with standard reference run simultaneously.
[b] Aspirin, phenacetin, and caffeine.
[c] Aspirin, phenacetin and caffeine citrate.

(±3.94%) when the conditions of chromatography were altered, e. g., different jars, lots of salted papers, humidity, and temperature. Therefore, it is recommended that a standard concentration of the known alkaloids be included within each experiment.

One advantage of this chromatographic method is that in the preparation of the samples, no elaborate extraction procedure with organic solvents is required. Tinc. chloroformis et morphinae co. preparation could be used directly. Other water extractable ingredients in these preparations did not interfere with the procedure. An example of a replicate analysis of an assay of an aspirin, phenacetin, caffeine, and codeine tablet (one-half grain of codeine phosphate per tablet) is given in Fig. 3. One excipient, a yellow dye, was found close to the point of origin of the chromatogram which is not visible in the photographic reproduction of Fig. 3.

SUMMARY AND CONCLUSIONS

The densitometric paper chromatographic procedure was applied to pharmaceutical preparations containing micro quantities (5–50 γ) of alkaloids with an average standard deviation of ±3.94 per cent. A simple preliminary sample preparation step is required. The use of a self-integrating densitometer enables the quantation to be achieved more rapidly than by usual procedures involving elution, etc. The procedure is applicable to a wide range of alkaloids and synthetic basic compounds. The type of compound affects the slope of the calibration curve. Application of the quantitative paper chromatographic method to the assay of small samples of narcotics received in legal cases and to the analysis of opium for purposes of determination of country of origin gave satisfactory results with the expenditure of a minimum of labor, time, and material.

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The Biological Action of Cellular Depressants and Stimulants I*

The Action of Ethyl Carbamate (Urethane) on the Endogenous Respiration and the Rate of Cell Division in *Tetrahymena pyriformis*

By JOHN J. EILER, JOSEPH Z. KREZANOSKI†, and KWAN-HUA LEE

The effect of urethane on the endogenous respiration and on the rate of cell multiplication has been studied in normal and in trained cells of *Tetrahymena pyriformis* with the view of obtaining information regarding the mode of action of narcotics.

THE CLASSICAL VIEW (1) that narcotics exert their characteristic biological effect through inhibition of cellular respiration persists at this time as one of several working hypotheses (2, 3). Despite the excellent studies by Quastel (4) and his associates, it has not been established that a depression of the respiratory activity in an oxygen-consuming cell is a primary, or even a necessary, event in the biological action of the narcotic or depressant drugs.

In studies on several classes of unicellular organisms, Fisher and co-workers (5–8) have shown that narcotics cause almost complete cessation of cell division at concentrations which depress cellular respiration only moderately. In studies on *Tetrahymena*, Ormsbee and Fisher (6) showed that urethane caused almost complete inhibition of cell multiplication at a concentration (0.1 *M*) which inhibited the endogenous respiration only about 25 per cent. Further, the regression line relating the rate of the endogenous respiration to an extended range of drug concentrations was such as to suggest the existence of two parallel resiratory systems with the more drug-sensitive system being geared to the endergonic processes of cell multiplication. Numerous studies (9–11) on a variety of biological forms are compatible with the view that growth and other energy-requiring processes may be geared to a specific fraction of the total respiration. Indeed, recent studies (12) on rat liver mitochondria suggest alternate pathways of electron transport, only one of which is phosphorylative and work promoting.

The successes which have been achieved (13, 14) in training organisms to grow in the presence of otherwise inhibitory concentrations of a drug suggested to us a possible means of putting to test the relationship suggested by Ormsbee and Fisher (6). Considerable support would be gained for the proposed gearing if, for example, organisms trained to grow in otherwise inhibitory concentrations of drug showed a concomitant decrease in sensitivity of a specific fraction of the respiration toward the depressing effects of the drug. Related to the question of the gearing between respiration and an endergonic process is the problem of whether the primary site of action of the drug is on respiration or on the endergonic process (3). A given set of results obtained in a training experiment, as just cited, could shed light on this vexing problem as well.

In the experiments reported here, we have studied the effects of urethane (ethyl carbamate) on the rate of cell multiplication and on the endogenous respiration in trained and untrained cultures of *Tetrahymena pyriformis*.

EXPERIMENTAL AND RESULTS

Materials and Methods.—The W strain of *Tetrahymena pyriformis* used in this study was graciously supplied by Prof. Daniel Mazia. Stock cultures were maintained in 300-ml. Erlenmeyer flasks containing 40 ml. of a medium consisting of 1.8% proteose-peptone (Difco) and 0.2% yeast extract (Difco). The cultures were maintained at 27° in the dark with weekly transfers. The sterility of both the stock and the experimental cultures was checked by microscopic observation and by plating on agar. Urethane was added to the medium as a sterile solution prior to inoculation. The appropriate concentration was sterilized by passage through a bacterial filter.

The turbidity measurements were made with a Klett-Summerson photoelectric colorimeter, using the No. 42 filter to reduce the influence of the colored medium. All turbidity values are reported in Klett units.

The procedure used for the counting of cells was essentially that of Hall, Johnson, and Loefer (15), using a Sedgewick-Rafter counting chamber (Scientific Glass Co., No. W4000) and a Whipple ocular micrometer. Cells were killed through addition of an aliquot of diluted formalin to yield a final concentration of 1% formaldehyde. When necessary, samples were diluted with a 0.24% solution of sodium chloride. The total number of cells counted in

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† Fellow of the American Foundation for Pharmaceutical Education. Present address: Medical College of Virginia, Richmond.

each sample was between 500 and 1,000. Recently Scherbaum (16) has established that the error in this counting procedure is about 5%.

The alkali-soluble biuret-detectable protein was determined on washed cell preparations using the procedure of Gornall, *et al.* (17). Cells were collected and washed with the above-mentioned solution of sodium chloride through the use of centrifugation at 2,500 r. p. m. (International Centrifuge, size 1) for five minutes. The biuret reagent was added directly to the cells washed free of medium and the absorbance was determined in a model B Beckman Spectrophotometer, using crystalline bovine albumin as a standard. The values so obtained were the same as when the washed cells were first treated with trichloroacetic acid and the precipitated protein washed with diluted trichloroacetic acid prior to the addition of the biuret reagent. The simpler of the two procedures was used since all values are comparative and not absolute.

Growth Studies.—All critical growth trials were carried out using 2,000-ml. Povitsky flasks containing 750 ml. of culture medium. The reasonably large surface-to-volume ratio thus obtained fostered rapid growth and the large volume permitted the frequent withdrawal of samples without marked changes in the surface-volume ratio of the culture. Further, in this fashion, the growth studies were conducted under the same conditions as were necessary to obtain sufficient cells for the respiration studies.

First, the effect of various concentrations of urethane on the rate of increase of cell mass was determined in order to insure that our strain of *Tetrahymena* behaved similarly to the one used by Ormsbee and Fisher (6). The results of such growth trials testing the effect of six concentrations of urethane are presented in Fig. 1. In these trials each flask, containing 750 ml. either of the medium or the medium with the indicated concentration of urethane, was inoculated with 5.0 ml. of a suspension (5×10^5 cells) from a stock culture in the rapid phase of growth. The flasks were incubated at 27° in the dark. Aliquots were withdrawn about every twelve hours for the determination of turbidity and cell protein. Only the results of the protein determinations are given in Fig. 1 since the Klett's units and the protein content were directly related throughout all growth trials with and without the several concentrations of urethane. The results, showing a graded effect of the several concentrations of the drug with essentially no growth at 0.11 *M*, are in good agreement with the findings of Ormsbee and Fisher (6).

To test the possibility of training the protozoa to overcome, at least partially, the growth-inhibiting effects of urethane, the organisms were subcultured in Klett-Summerson tubes containing 5.0 ml. of either 2.0% proteose-peptone (Difco) or the same medium with urethane. The 0.1 ml. of inoculum was taken from a proteose-peptone culture in the stationary phase of growth. The tubes were incubated in the dark at 25°. Two-hundredths *M* urethane was selected as the concentration for initial exposure of the cells to undergo training, since at this concentration growth is only slightly inhibited or even stimulated. Both normal cells and the cells undergoing training were subcultured every five days. The cells under training were transferred

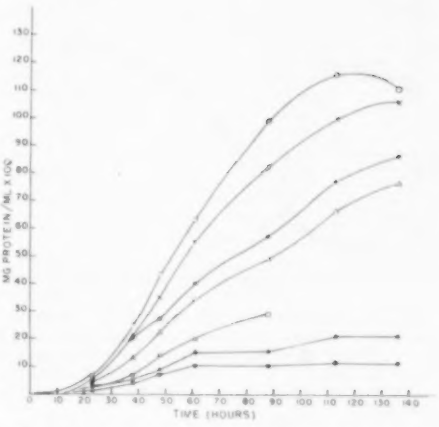

Fig. 1.—The effect of several concentrations of urethane on the growth of normal cultures: ○ = control; X = 0.03 *M*; ● = 0.056 *M*; Δ = 0.07 *M*; □ = 0.09 *M*; ▲ = 0.10 *M*, and ■ = 0.11 *M*. The ordinate represents the experimental value times 100.

twice into medium containing 0.02 *M* urethane and twice into medium containing 0.04 *M* drug. The normal or control cells were subcultured with the same frequency.

To test the results of training, the time-dependent increases in turbidity of the normal cells in the presence and in the absence of 0.08 *M* urethane were compared with the increases of the drug-exposed cells in the presence of 0.04 *M* and 0.08 *M* urethane. The results presented in Fig. 2 indicate that the cells trained in 0.04 *M* drug increased in mass at about the same rate in the presence of either 0.04 *M* or 0.08 *M* urethane as did the normal cells in the absence of the drug. The cells previously unexposed to the drug showed a greatly retarded rate of increase of mass in the presence of the 0.08 *M* urethane. Clearly, training under these conditions leads to an almost complete reduction in the sensitivity to the growth-inhibiting effects of 0.08 *M* urethane. The pronounced lag in growth observed in all trials in Fig. 2 is due to the use of mature cultures as inocula in the subculturing and in the testing, and is not due to the action of the drug.

Similar studies indicated that concentrations above 0.08 *M* urethane could not be used for testing. When the training concentration was raised to 0.1 *M* only meager growth was observed for several subculturings, followed by complete failure of growth.

It was necessary to alter the conditions for training to provide sufficient uniform material for the later studies. All subsequent subculturing made use of 300-ml. Erlenmeyer flasks containing 40 ml. of medium with or without urethane. To permit more rapid growth than that indicated in Fig. 2, the medium consisted of 1.8 proteose-peptone and 0.2% yeast extract, as in the stock cultures.

Trials testing the effectiveness of training under the new conditions were carried out using turbidity as the index of cell mass. The results of one set of trials are presented in Fig. 3. The cells undergoing training were brought through a series of concentra-

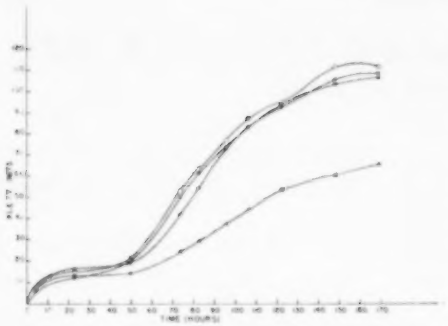

Fig. 2.—The effect of urethane on the growth of test tube cultures of trained and untrained cells: ○ = control; ● = trained in 0.04 *M* urethane and tested in 0.04 *M*; X = trained in 0.04 *M* urethane and tested in 0.08 *M*; ▲ = untrained and tested in 0.08 *M*.

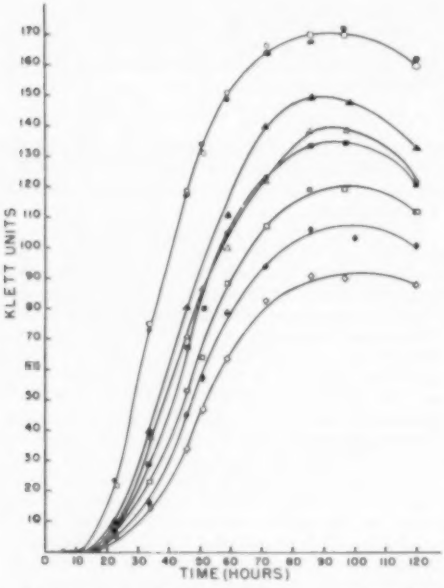

Fig. 3.—The effect of urethane on trained and untrained cells which had been cultured in Erlenmeyer flasks containing the standard medium. Normal cells, ○ = control; △ = 0.07 *M*; □ = 0.08 *M*, and ◇ = 0.09 *M*. Trained cells, ● = control; ▲ = 0.07 *M*; ■ = 0.08 *M*, and ◆ = 0.09 *M*.

tions of urethane and, at the time of the growth trials, had been subcultured in 0.08 *M* urethane every forty-eight hours for four times (two hundred sixteen hours). Control cells were subcultured in the plain medium with the same frequency. The growth trials were carried out in 300-ml. Erlenmeyer flasks fitted with a side arm suitable for insertion into the Klett-Summerson instrument. Forty ml. of medium or medium with urethane were inoculated with 0.5 ml. of suspension from either of the two final subcultures.

The results given in Fig. 3 indicate that subculturing and testing under conditions which permit greater oxygenation (surface-volume ratio) and more rapid growth do not give rise to so great a decrease in sensitivity to the drug as was observed in the results presented in Fig. 2. All three test concentrations of urethane produced a proportional decrease in the rate of increase in cell mass. However, there remains a significant effect of training, since in each test concentration the drug exposed cells multiplied (turbidity) more rapidly than the previously unexposed cells.

Similar studies demonstrated that shorter periods of training in 0.08 *M* urethane produced no measurable increase in drug resistance. Extension of the training period, with frequent subculturings, during fifteen days, or even six months, did not give better results than those presented in Fig. 3. In no case did training permit growth in the presence of 0.1 *M* urethane.

To observe the effect of training on increase in cell mass as judged both by turbidity and cell count, growth trials were conducted in Povitsky flasks. The 750 ml. of medium with or without urethane were inoculated with either trained or normal cells to yield an initial density of about 1,000 cells per ml. The trained cells had been subcultured in 0.08 *M* urethane every forty-eight hours for fifteen days. The normal cells were subcultured with the same frequency. Samples were withdrawn approximately every twelve hours during the incubation period.

To conserve space, the results of the influence of the drug on the turbidity of the cultures are presented in Fig. 4 only as a regression line correlating turbidity with cell count. The cell count and Klett reading for every sample taken from both the trained and untrained cell cultures were used in the preparation of Fig. 4. While several factors undoubtedly contribute to the observed lack of linearity in the regression line, the principal factor relates to dead cells and debris. As the cultures increase in age, the number of dead cells and the debris contribute to the Klett readings in a manner not reflected in the cell counts which included principally live cells. It is significant to point out that the results from both types of cells fit the curve equally well. This fact precludes the need to present both cell count and turbidity data and suggests (18) that there is no great disparity in size between the normal and the trained cells. Closer attention was not given to the effect of training on cell size and morphology.

The results of the effect of the drug on the rate of increase in cell count are presented in Fig. 5 as a semilog curve, wherein the time-dependent count divided by the initial count is plotted on a logarithmic scale against time. It is clear from Fig. 5 that both the trained and the untrained cells multiplied at an almost identical rate in the absence of drug, while the trained cells multiplied more rapidly in each of the test concentrations of drug. Thus, training produced an increase in drug resistance as judged both by turbidity (cell mass) and cell count. The lack of linearity which develops at about the fifth generation is related to oxygen want and will be considered more fully in the second paper in this series (19). The period of logarithmic growth, as is

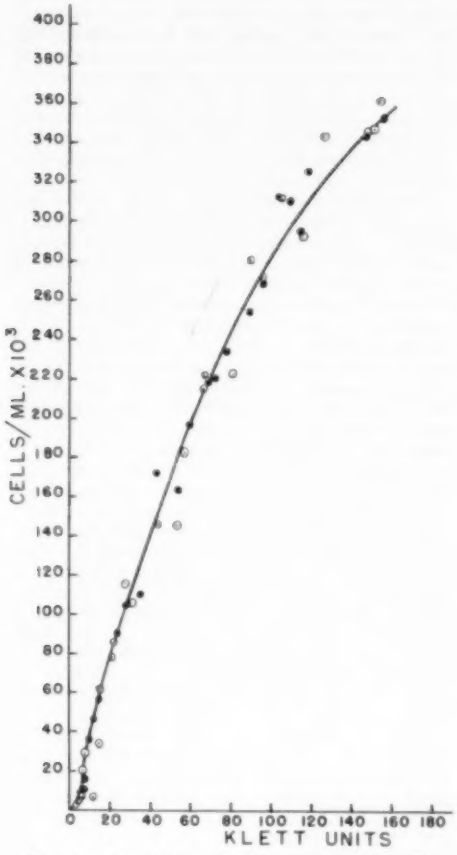

Fig. 4.—Correlation of cell numbers with Klett readings. Open circles relate to normal cells; solid circles relate to trained cells. The values on the ordinate are to be multiplied by 1,000 to yield the experimental value.

customary, has been used to calculate the mean generation time, as given in Table I, to provide quantitative estimates of the effect of training. The values for the per cent increase in mean generation time presented in Table I indicate that each of the test concentrations have about one-half the inhibitory effect on the trained cells as upon the untrained cells.

Respiration Studies.—The effect of various concentrations of urethane on the rate of the endogenous respiration of normal and trained cells was measured at 27° essentially according to the procedure of Ormsbee and Fisher, using the conventional Warburg technique. The drug-exposed cells had been subjected to weekly subculturing in 0.08 *M* urethane for the period of approximately six months. To obtain sufficient cells, the final growth was conducted in Povitsky flasks. The normal cells were harvested from the 750 ml. of medium at the end of forty-eight hours while the trained cells were harvested from the 750 ml. of urethane-containing medium at the end of seventy-two hours. In this

TABLE I.—THE EFFECT OF ETHYL CARBAMATE ON THE GENERATION TIME OF NORMAL AND TRAINED *Tetrahymena*

Cell Type	Molar Concn. of Urethane	Mean Generation Time[a] Time, Hr.	Increase, %
Normal	0.00	3.4	..
	0.07	4.4	29
	0.08	5.0	47
	0.09	5.6	65
Trained	0.00	3.4	..
	0.07	4.0	18
	0.08	4.3	26
	0.09	4.7	38

[a] The mean generation times were calculated from the first-order rate constants estimated from the slopes of the curves in Fig. 5.

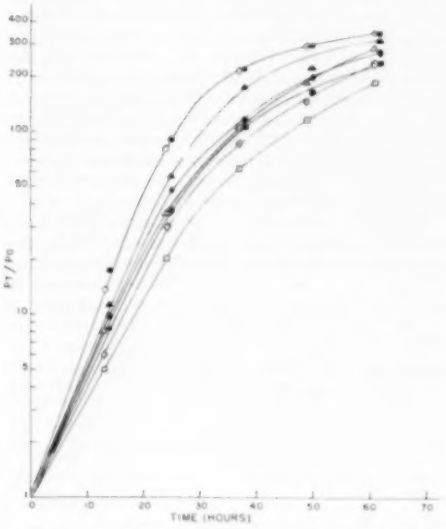

Fig. 5.—The effect of several concentrations of urethane on trained and untrained cells. The time-dependent cell count (P_T) divided by the initial cell count (P_0) is plotted on a logarithmic scale. Normal cells, ○ = control; Δ = 0.07 *M*; ◇ = 0.08, and □ = 0.09 *M*. Trained cells, ● = control; ▲ = 0.07 *M*; ◆ = 0.08 *M*, and ■ = 0.09 *M*.

way, approximately the same number of cells were obtained from each population. The cells were washed three times with 0.005 *M* phosphate buffer (pH 6.9) through the use of an angle head centrifuge. Each centrifugation was carried out for three minutes at 150 × g. The washed cells were suspended in 30 ml. of the phosphate buffer to yield a suspension containing about 2.5 mg. of cell protein per ml. (1.7×10^6 normal cells per ml.). After about twenty minutes of time for preparation, the Warburg vessels were loaded with 1.0 ml. of the cell suspension, to which was added 0.5 ml. of the appropriate concentration of urethane in phosphate buffer, additional phosphate buffer was added to give a final volume of 2.3 ml. in the main compartment in all vessels. The center well was charged with alkali (0.2 ml. 20% KOH) and filter paper in the usual manner. After a ten-minute period for temperature equilibrium, the respiration trials were carried out

with air as the gas phase and with shaking at the rate of 130–134 cm. excursions per minute. It was established that the values reported were not limited by the rate of diffusion of oxygen. The uptake of oxygen was measured for a period of either sixty or ninety minutes, during which time the rate was essentially constant except in concentrations in excess of 0.2 *M* wherein the rate fell markedly and the effect of the drug was not reversible.

The rate (μl. O_2/hour/mg. protein) of the endogenous respiration of the normal cell amounted to 23, while that of the trained cell amounted to 34. These almost identical values are in good agreement with values in the literature reported on a dry weight basis (20, 21, 22) when allowance is made for the difference in the manner of expressing the values. Only one set of the many trials carried out is reported in the data presented in Fig. 6. Each point on the sets of curves in Fig. 6 represents the average of the results from two vessels from each of two cultures. Ten concentrations of urethane, ranging from 0.025 *M* to 0.2 *M*, were studied in addition to the non-urethane controls. The results presented in Fig. 6 are in the form of the "mass-action law" plot used by Ormsbee and Fisher: *U* is the rate of oxygen consumption in the presence of a given concentration of urethane; *I* is the difference between *U* and the respiration in the absence of urethane.[1]

As first pointed out by Ormsbee and Fisher, the effect of urethane on the rate of the endogenous respiration is best characterized by two intersecting straight lines. In the case of both classes of cells, the lines intersect at a urethane concentration of about 0.11 *M*. In the results of Ormsbee and Fisher, the break in the curve was observed at 0.1 *M* urethane. The difference in the sensitivity to the drug of the more sensitive respiration, as indicated in Fig. 6, is small. At 0.11 *M* urethane the endogenous respiration is depressed 26%, while the respiration of the trained cell is depressed 35%. The differences are less at lower concentrations. At the lowest concentration, the difference amounts to about 4%. In any event, the trained cell is more, rather than less, sensitive to the respiratory depressing effects of urethane. This finding is in contrast to the effects on growth, wherein the trained cell is significantly less sensitive.

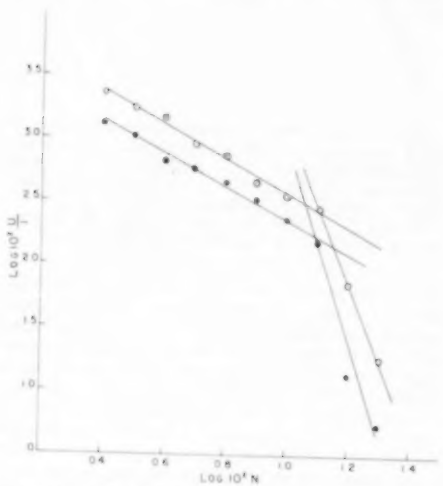

Fig. 6.—The effect of urethane on the endogenous respiration of trained and untrained cells. The data are plotted according to the formulation of Ormsbee and Fisher (see text).

[1] According to Fisher (11), $\log (U/I) = \log K - a \log N$, where K is the dissociation constant of a drug-enzyme complex, N is the concentration of drug, and a is the number of molecules of drug uniting with each active enzyme site.

Metabolism of Drug.—While these studies had little concern with the mechanism by which the increase in drug resistance took place, it was deemed necessary to exclude the possibility that the improved growth was due to the circumstance that the trained cells metabolized urethane faster and thus caused a reduction in the relative concentration. Due to the greater exposure of the cells to urethane in the growth trials, in contrast to the respiration trials, an increased capacity to metabolize urethane would bias the growth results more than the respiration data. Accordingly, we tested the ability of the trained cell to metabolize urethane.

An amount of urethane to yield a final concentration of 0.08 *M* was added to each of four 750-ml. portions of medium and to each of two 750-ml. volumes of water. Two of the flasks containing medium were inoculated with trained cells and incubated for seventy-two hours, after which the cells were removed by centrifugation. One hundred-milliliter aliquots were then removed from each of the cell-free media and from each of the remaining vessels and extracted repeatedly with ethyl ether by a standardized procedure. The urethane in each of the ether extracts was crystallized with the use of petroleum ether, dried, and weighed. The averages of the duplicate weights from each set of aliquots were: 475 mg. from water; 475 mg. from the plain medium, and 480 mg. from the medium which had supported cell growth. The melting points of the several samples were in the range 48–50°. The agreement in amounts recovered by the standardized procedure indicates that degradation of urethane is not significant and that it is not the mechanism by which training in growth takes place.

DISCUSSION

The results presented in Figs. 1 and 6, pertaining to the control cells, establish the fact that despite some differences in experimental conditions, the strain of *Tetrahymena* used in these experiments responded to urethane similarly to the strain used by Ormsbee and Fisher (6). As in their results, the "mass-action law" plot of the results relating the rates of endogenous respiration to the concentration of urethane is best represented by two intersecting straight lines (Fig. 6). Likewise, the concentration of drug associated with the point of intersection is the same as that which inhibited cell multiplication almost completely. According to them, the respiratory activity suggested by the line with the lesser slope is related in a specific fashion to the process of cell multiplication. The respiratory function represented by the greater slope was considered to be associated with the vegetative activity of the cell. The argument was advanced by us (to present only one possibility) that support for their proposal would be obtained if cells trained to grow in urethane

showed an appropriate decrease in the sensitivity of the respiration to the depressing effect of the drug. The significance of the results would be enhanced if the change in sensitivity were confined to that portion of the respiration suggested by the lesser slope.

The results presented in Figs. 2, 3, and 5 demonstrate clearly that training in urethane promoted measurable increases in the resistance of the cultures to the growth-inhibiting effect of the drug. However, the results obtained under conditions of poor oxygenation and slow growth (Fig. 2) stand in sharp contrast to the results obtained under conditions of good aeration and more rapid growth. The cell trained in 0.04 *M* urethane under conditions of poor oxygenation developed a complete insensitivity to the growth-inhibiting effect of 0.08 *M* urethane. It is noteworthy that even with the degree of training established relative to the effect of 0.08 *M* urethane, it was not possible to train the organisms to grow, even slowly, in 0.1 *M* urethane. The limited degree of training which we have achieved with *Tetrahymena* is in marked contrast to the successes achieved by Hinshelwood (13) and co-workers working with bacteria. It is generally recognized that protozoa adapt to the inhibitory effect of most drugs only slowly and not extensively (23).

The results presented in Fig. 6 indicate definitely that neither the more sensitive respiration nor the total endogenous respiration showed a decrease in drug sensitivity as a result of training. Indeed, the adapted cells were slightly more sensitive to the respiratory effect of the drug.

These findings indicate quite definitely that there is no change in respiration, reflected in the endogenous respiration, that would account for the degree of training obtained in the growth studies. In a similar fashion, Preston and Eiler (24) could find no decrease in sensitivity to the respiration depressing effect of pentobarbital in brain slices from rats rendered tolerant to the action of the drug. However, McCashland (25) recently observed an adaptive increase in the respiration, measured under the conditions of growth, in *Tetrahymena* trained to resist the effects of potassium cyanide.

The absence of a rigid coupling between respiration and cell multiplication, however, does not preclude a significant relation between the two processes. An important aspect of cell respiration, together with the anaerobic metabolism, is the supply of energy it provides for endergonic processes. The energy-rich phosphate compounds formed as a result of aerobic oxidations, and to a lesser extent anaerobic oxidations, make possible growth and division of the cell. A coupling between respiration and growth based upon supply and demand for energy-rich compounds is a necessity in the life of aerobic organisms. A significant depression in respiration must be accompanied by a decline in endergonic processes unless compensated by an increase in the rate of the reactions which supply energy under anaerobic conditions. It is possible that just such an increase in anaerobic metabolism may be basic to the improvement in growth observed in our experiments.

The greater resistance to the growth-inhibiting effects of urethane established under the conditions of poor oxygenation and slow growth (Fig. 2) suggests that the principal change in the trained cell is the development of a more significant anaerobic metabolism. Ryley (21) has shown that *Tetrahymena pyriformis* is able to survive and maintain motility under anaerobic conditions. The anaerobic metabolism, at least of mammalian cells, is not nearly so sensitive to the inhibitory effects of narcotics as is the aerobic metabolism; indeed, the aerobic glycolysis of brain is increased by narcotics (4). An increase in anaerobic metabolism is a logical consequence of subculturing in relatively anaerobic conditions, especially when the aerobic metabolism has been depressed somewhat by urethane.

The acceptance of an adaptive change in the anaerobic metabolism carries with it the idea that growth and division are inhibited primarily through the action of the drug on respiration. Certainly, if the growth-inhibiting effects are, in part, reversed by the development of an anaerobic metabolism, it is not reasonable that that fraction of the growth could have been inhibited by a direct effect of the drug on the growth process *per se*.

It should be clear that the respiration whose inhibition has been considered to lead to an inhibition of growth is not the respiration which has been measured in these experiments; rather, it is the respiration which is measured under conditions in which cell division is taking place. The possible relation between the two respirations, relative to growth, is not known at this time. Fortunately, such interrelations, together with the behavior of the anaerobic reactions, are susceptible to experimental study.

SUMMARY

1. The effects of urethane on the endogenous respiration and on the rate of cell multiplication has been studied on trained and untrained cells of *Tetrahymena pyriformis* with the view of gaining some understanding of the mode of action of narcotic drugs.

2. The results of the action of the drug on the untrained cells are in good agreement with the earlier findings of Ormsbee and Fisher which suggested a significant relation between cell multiplication and respiration.

3. The cells from cultures which had been trained by repeated subculturing in the drug showed a higher rate of cell multiplication in a given concentration of drug than did the untrained cells. The difference was most marked in cultures which have been trained under conditions of poor oxygenation. However, the endogenous respiration of the trained cells in a given concentration of urethane was no higher than that of the untrained cells.

4. The results are considered in terms of a possible mode of action of urethane.

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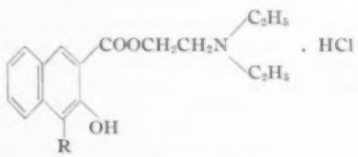
Dialkylaminoethyl Esters of 4-Nitro- and 4-Amino-3-hydroxy-2-naphthoic Acids*

By GEORGE M. SIEGER†, WILLIAM M. ZIEGLER†, DAVID X. KLEIN, and HERMAN SOKOL

The 2-diethylaminoethyl ester of 3-hydroxy-2-naphthoic acid was nitrated in acetic acid solution to yield the corresponding ring-substituted 4-nitro derivative. Catalytic hydrogenation of the nitro compound gave 2-diethylaminoethyl 4-amino-3-hydroxy-2-naphthoate hydrochloride. The anesthetic properties of these nitro and amino derivatives have been studied and compared with unsubstituted 3-hydroxy-2-naphthoic acid esters of the same series.

IN A PREVIOUS PUBLICATION (1) the synthesis of a number of dialkylaminoalkyl esters and amides derived from 3-hydroxy- and 3-alkoxy-2-naphthoic acids and some of their physiological properties were described. In continuation of these earlier studies, it was deemed worthwhile to prepare ring-substituted nitro and amino derivatives of the 2-diethylaminoethyl ester of 3-hydroxy-2-naphthoic acid in order to determine whether or not the anesthetic activity could be enhanced with relation to the unsubstituted ester. The rationale supporting the possibility for an increased anesthetic effect lies in the fact that the proposed compounds could be considered naphthoic acid analogs of ring-substituted local anesthetics, such as procaine (2-diethylaminoethyl 4-amino-1-benzoate) and Naphthocaine (2-diethylaminoethyl 4-amino-1-naphthoate) and their related 4-nitro compounds (3, 4). Since it has been demonstrated in several series of the local anesthetics that the introduction of second ring substituents, such as amino or alkoxyl groups, sometimes increases the activity (3–9), there seemed to be a sufficiently good reason for preparing a ring-amino derivative of 2-diethylaminoethyl 3-hydroxy-2-naphthoate for testing as a local anesthetic.

Synthesis of the proposed ring-substituted amino derivative of 2-diethylaminoethyl 3-hydroxy-2-naphthoate was accomplished by nitrating the hydrochloride of this ester (I) in glacial acetic acid under the same conditions described by Gradenwitz (2) for the preparation of methyl 4-nitro-3-hydroxy-2-naphthoate (V) and subsequent reduction of the nitro derivative (II) to the corresponding amino compound (III) by catalytic hydrogenation.



II – R = NO_2
III – R = NH_2

Designation of the nitro and amino groups as ring substituents in the 4-position is consistent with the work of Gradenwitz (2) who obtained the 4-nitro ring derivative of the methyl ester of 3-hydroxy-2-naphthoic acid (V) under conditions similar to those used for nitration of the diethylaminoethyl ester. These structures were established further by hydrolyzing both the nitrated methyl and 2-diethylaminoethyl esters (V and II)

* Received September 24, 1958, from the Research Laboratories of the Heyden Newport Chemical Corp., Garfield, N. J.
† Present address: Lederle Laboratories, American Cyanamid Co., Pearl River, N. Y.
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to give the same acid, namely, 4-nitro-3-hydroxy-2-naphthoic acid (VI) described by Robertson (10).

PHARMACOLOGY

In comparative rabbit corneal anesthetic tests run according to the method of Sollmann (11) and described more fully in our previous publication (1), the 4-nitro and 4-amino ring-substituted derivatives of 2-diethylaminoethyl 3-hydroxy-2-naphthoate hydrochloride (II and III) did not appear to be significantly more active than the unsubstituted ester (I) and were less active than the homologous 3-diethylaminopropyl 3-hydroxy-2-naphthoate hydrochloride (Bonacaine-G) (1).

EXPERIMENTAL

2-Diethylaminoethyl 3-Hydroxy-2-naphthoate Hydrochloride (I).—This ester was prepared by the reaction of 3-hydroxy-2-naphthoic acid with the free base of diethylaminoethyl chloride as described in procedure C of our previous publication (1).

2-Diethylaminoethyl 4-Nitro-3-hydroxy-2-naphthoate Hydrochloride (II).—Thirty-two grams (0.1 mole) of 2-diethylaminoethyl 3-hydroxy-2-naphthoate hydrochloride (I) was suspended in 350 ml. of glacial acetic acid. While the suspension was chilled in an ice bath and stirred, 10 ml. (*ca.* 0.15 mole) of concentrated nitric acid, diluted with 150 ml. of glacial acetic acid, was slowly added dropwise so that the temperature remained below 20°. After all the nitrating solution had been added the ice bath was removed. The temperature of the reaction mixture rose slowly to 29° and its color turned from pale yellow to dark orange. An additional 100 ml. of glacial acetic acid was added to the stirred mixture. After about twelve hours the mixture was filtered through a sintered-glass funnel to remove the somewhat pasty crude mononitro compound. After triturating the crude product with 2B ethanol and heating it under reflux with 250 ml. of hot anhydrous ethanol for several hours, the product was filtered. The yellow crystalline compound was triturated repeatedly with alcohol-ether; m. p. 219–221°; yield, 13 Gm. (35.4%).

Anal.—Calcd. for $C_{17}H_{21}O_4N_2Cl$: N, 7.6; Cl, 9.6. Found: N, 7.3; Cl, 9.5.

2-Diethylaminoethyl 4-Amino-3-hydroxy-2-naphthoate Hydrochloride (III).—Twelve grams (0.0326 mole) of 2-diethylaminoethyl 4-nitro-3-hydroxy-2-naphthoate hydrochloride (II) and 0.3 Gm. of platinum oxide catalyst were suspended in 150 ml. of ethanol and 10 ml. of water. The mixture was hydrogenated at room temperature, under an initial pressure of 44 p.s.i., for six hours. After removal of the catalyst, the mixture was concentrated *in vacuo* at as low a temperature as possible. On chilling the concentrate, gold-yellow needles separated from the solution. After filtration, the dried product melted 184.8 to 185.8°; yield, 2.5 Gm. (22.5%). It was soluble in water and much less soluble in small volumes of cold alcohol or acetone.

Anal.—Calcd. for $C_{17}H_{23}O_3N_2Cl$: C, 60.3; H, 6.8; N, 8.3; Cl, 10.5. Found: C, 60.4; H, 7.0; N, 8.4; Cl, 10.3

Methyl 3-Hydroxy-2-naphthoate (IV).—The procedure used for preparing the methyl ester of 3-hydroxy-2-naphthoic acid was essentially a modification of the procedure of Cohen and Dudley (12). The yellow crystalline ester obtained melted 71 to 73°. Literature (12) gives 73 to 74°.

Methyl 4-Nitro-3-hydroxy-2-naphthoate (V).—The methyl ester of 3-hydroxy-2-naphthoic acid (IV) was nitrated in acetic acid by essentially the same procedure as described by Gradenwitz (2). The mononitro compound was isolated as yellow crystalline needles melting 186 to 189° in a 30% yield. The literature (2) gives 188 to 189° as the m. p. for this compound.

Anal.—Calcd. for $C_{12}H_9O_5N$: N, 5.68; ester value, 227 mg. KOH/Gm. Found: N, 5.69; ester value, 234.9 mg. KOH/Gm.

4-Nitro-3-hydroxy-2-naphthoic Acid (VI).—Twenty grams (0.081 mole) of methyl 4-nitro-3-hydroxy 2-naphthoate (V) was saponified with 400 ml. of 10% sodium hydroxide solution diluted with 400 ml. of water over a two-hour period with the aid of gentle heating until almost a completely red-colored solution was obtained. While hot, the solution was filtered. The small amount of insoluble material on the filter plate was washed with an additional 200 ml. of water. The red-colored filtrate was acidified with excess concentrated hydrochloric acid (*ca.* 300 ml.), causing the color of the filtrate to turn yellow and the precipitation of a yellow-colored product. It was filtered, washed with water, and dried; yield, 16.4 Gm. (87%); m. p. 233.9 to 234.9° Robertson (10) reported 233 to 238° (decompn.) as the melting point for this compound.

Anal.—Calcd. for $C_{11}H_7O_5N$: N, 6.02; mol. wt., 233. Found: N, 5.74; mol. wt., 231 (by potentiometric titration).

Hydrolysis of 2-Diethylaminoethyl 4-Nitro-3-hydroxy-2-naphthoate Hydrochloride (II).—Following the same procedure as described above, 2-diethylaminoethyl 4-nitro-3-hydroxy-2 naphthoate hydrochloride (II) yielded the same corresponding acid (VI), as ascertained by melting point and microanalytical determinations.

SUMMARY

1. The 4-nitro and 4-amino ring derivatives of 2-diethylaminoethyl 3-hydroxy-2-naphthoate hydrochloride have been synthesized.

2. Introduction of the 4-nitro and 4-amino substituents into the 3-hydroxy-2-naphthoic acid nucleus of the basic ester did not alter its anesthetic activity significantly.

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In Vivo Fibrinolytic Effect of Various Proteolytic Enzymes: Quantitative Tests Employing Iodine^{131}-Labeled Clots*

By J. L. AMBRUS†, N. BACK†, S. GOLDSTEIN††, C. M. AMBRUS†, and J. W. E. HARRISSON

Purified fibrinogen was labeled with I^{131}. Clots were produced in various blood vessels of dogs with the aid of this material. Radioactivity was continuously registered over the clot. Thus, the clot dissolving activity of potential fibrinolytic agents could be evaluated quantitatively. Four proteolytic enzymes of animal and plant origin were found to be ineffective as fibrinolytic agents.

MANY METHODS have been described to produce experimental thrombosis in animals. In the study of potential fibrinolytic agents, the disappearance of the clot was observed mainly by palpation, visual observation during autopsy, or histologic preparations. All these methods are obviously qualitative. We have developed methods (1, 2) for the quantitative testing of such agents. Fibrinogen was labeled with I^{131} and the radioactivity of the clot produced with this material *in vivo* was continuously recorded over the thrombosed area. Using this method together with conventional procedures, the fibrinolytic activity of trypsin and various preparations of plasmin have been tested. Preparations and dose ranges of plasmin were found which effectively dissolved clots without causing important biochemical changes (3). Nevertheless, because of various difficulties involved in the use of such preparations therapeutically, it was felt that a preliminary screening should be undertaken in search for other effective agents. Four proteolytic enzymes of animal or plant origin were tested in this study: crude pancreatic protease, ficin, papain, and carboxypeptidase.

MATERIALS AND METHODS

Adult mongrel dogs of both sexes were used. They were anesthetized with 25 mg./Kg. sodium pentobarbital; this dose was supplemented with further doses as needed. The veins or arteries to be thrombosed were prepared and the segment to be used was isolated between two artery clamps. All side branches were ligated except for one which was cannulated with a polyethylene tube. Most of the blood from the isolated segment was withdrawn through this cannula into a syringe containing 0.1 to 0.2 ml. I^{131}-labeled fibrinogen. The mixture was withdrawn and reinjected three times to insure proper mixing. This was followed by the introduction of thrombin in 0.1 ml. saline.

A semi- or completely constricting ligature was placed distal (in arterial clots) or proximal (in venous clots) to the thrombosed area. This served to prevent escape of the clot without being lysed. If such escape nevertheless occurred, the embolic clot particles could easily be detected by scanning the animal with the scintillation counter. Experiments in which this occurred were excluded from evaluation. It is possible that in some studies reported by other investigators in which conventional methods were used, escape of the clot may have been recorded as lysis of the clot. This may explain some positive results in the literature which could not be confirmed by this method.

After about thirty minutes the polyethylene cannula was removed and the side branch ligated; the artery clamps were removed and the vessel was placed in the trough of a specially constructed lead shield which accommodated a scintillation counter. This was connected to a radiation rate meter which, in turn, was connected to a counter as well as to an Esterline recorder. The former registers the total number of counts within any desired time period, while the latter continuously graphs count per minute values. All values in the table refer to radioactive counts per minute.

In preliminary experiments it was shown that decrease in radioactivity registered by these methods is proportional to lysis of the clot. After lysis of the clot with trypsin, which was incorporated into the clot at the time of its formation, radioactivity rapidly decreased in the blood and did not increase to any important degree in any organ. Most of the I^{131} was recovered from the urine. Probably I^{131}-labeled fibrin was broken down to small molecular size peptides which are not taken up by the thyroid gland.

Radioactivity was continuously recorded for at least four hours, and every twenty-four hours thereafter. The dogs received 1 million units penicillin and 250 mg. streptomycin subcutaneously daily, and veterinary Terramycin powder locally. The wound was closed with wound clips and reopened for the daily recordings. In the tables, the values before administration of the agents to be tested and at four, twenty-four, and ninety-six

* Received May 5, 1955, from the LaWall Memorial Laboratory of Pharmacology and Biochemistry, Department of Pharmacology, Graduate School, Philadelphia College of Pharmacy and Science, Philadelphia 4, Pa.

† Present address: Roswell Park Memorial Institute, Buffalo, N. Y.

†† Present address: Norwich Pharmaceutical Co., 17 Eaton Ave., Norwich, N.Y.

We are greatly obliged to Parke, Davis and Co. for thrombin and Benadryl; Lederle Laboratories for penicillin; Sharp and Dohme Division of Merck and Co. for streptomycin; Chas. Pfizer and Co. for Terramycin, and Warner-Chilcott Labs. for Simplastin.

hours were reported. If the animal died before the end of one of these periods, the values were recorded in the tables at the nearest of these time periods.

As an additional criterion of fibrinolysis, visual observation by transillumination was undertaken. The light source of a Metro photoelectric tensiometer was dismounted. This provided a powerful light source at the end of a thin, curved, stainless steel tube. By holding the light source under the thrombosed segment of the vessel, the presence or absence of fibrin network could be established to some degree. The following arbitrary units were used to report these data: 3—full clot, no fluid phase; 2—full clot, little fluid phase; 1—decreased clot size, more fluid phase; 0—no clot. Figure 1D[1] is a photograph of the experimental setup.

Iodine^{131}-labeled fibrinogen was prepared on the basis of a modification of the method of Mihalyi and Laki (4) as described previously (1, 2). Two grams of commercial bovine fibrinogen was dissolved in 100 ml. of a 0.1 *M* phosphate buffer at pH 6.4. To this was added 100 ml. cold distilled water. This was refrigerated for at least six hours. This was then centrifuged and the sediment discarded. To the supernatant was added a saturated solution of

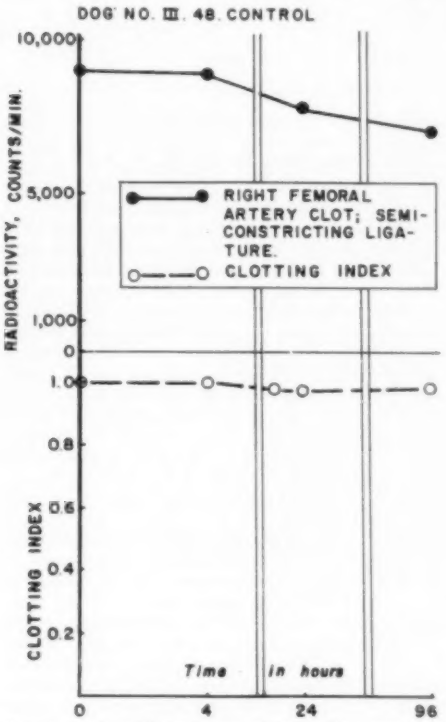

Fig. 1.—Effect of saline on clotting index and I^{131}-labeled clot in dog.

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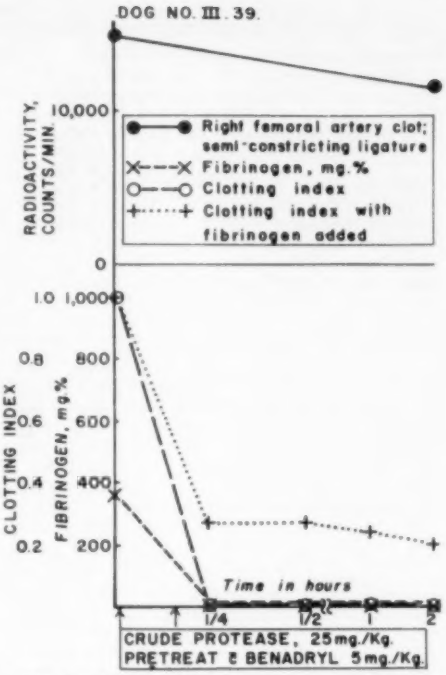

Fig. 2.—Effect of crude protease, 25 mg./Kg., on clotting index, fibrinogen level, and I^{131}-labeled clot in dog pretreated with Benadryl, 5 mg./Kg.

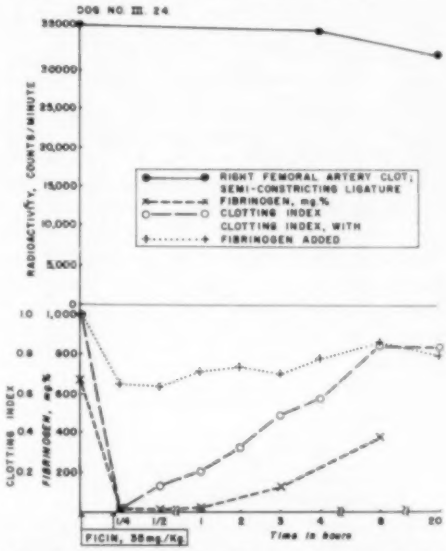

Fig. 3.—Effect of ficin, 35 mg./Kg., on clotting index, fibrinogen level, and I^{131}-labeled clot in dog.

ammonium sulfate equal to $^1/_3$ of the original volume resulting in a final concentration of 25% $(NH_4)_2SO_4$ which caused the precipitation of fibrinogen. This was then centrifuged and the precipitate was dis-

TABLE I.

Drug and Dose	Time	No. of Clots	Clots Showing a Decrease in Radioactivity of 0–25%	26–50%	51–75%	76–100%
Control	Up to 4 hr.	25	100.0	0.0	0.0	0.0
	Up to 24 hr.	26	88.5	7.7	3.8	0.0
	Up to 96 hr.	22	63.5	27.2	9.2	0.0
Carboxypeptidase, 21.7 mg./Kg.	Up to 4 hr.	3	66.7	33.3	0.0	0.0
	Up to 24 hr.	3	66.7	33.3	0.0	0.0
	Up to 96 hr.	3	0.0	66.7	0.0	33.3
Papain, 35 mg./Kg.	Up to 4 hr.	6	50.0	50.0	0.0	0.0
Ficin, 35 mg./Kg.	Up to 4 hr.	6	83.3	16.7	0.0	0.0
Crude protease, 25–35 mg./Kg.	Up to 24 hr.	3	66.7	33.3	0.0	0.0
	Up to 4 hr.	6	66.7	33.3	0.0	0.0

TABLE II.

Drug and *in vivo* Dose	*In vitro* Proteolytic Activity Loomis u./mg.	Radioactivity of Clot: Up to 4 hr. No. of clots	Up to 4 hr. Mean % lysis	Up to 24 hr. No. of clots	Up to 24 hr. Mean % lysis	Up to 96 hr. No. of clots	Up to 96 hr. Mean % lysis
Control saline	. .	25	6.6	26	13.9	22	25.3
Carboxypeptidase, 21.7 mg./Kg., single infusion	<0.01	3	15.8	3	19.5	3	30.0
Crude pancreatic protease, single infusion							
25 mg./Kg.	53.0	3	23.0				
35 mg./Kg.		3	27.3				
Papain, 35 mg./Kg., single infusion,	0.06	6	19.0				
Ficin 35 mg./Kg., single infusion	0.3	6	18.3	3	19.2		

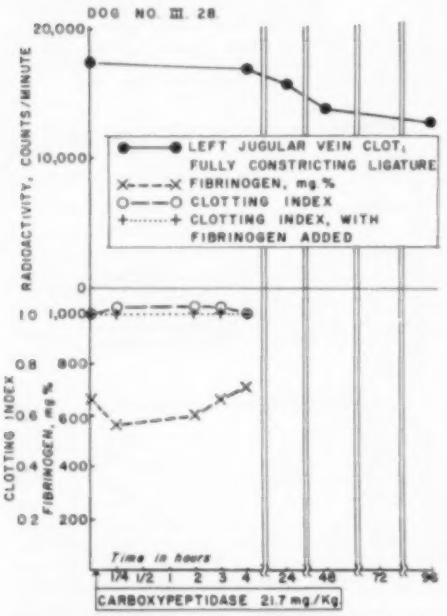

Fig. 4.—Effect of carboxypeptidase, 21.7 mg./Kg., on clotting index, fibrinogen level, and I^{131}-labeled clot in dog.

solved in 10 ml. of distilled water and dialyzed for twenty-four hours against 0.3 *M* KCl solution in the cold. A 0.1 *N* iodine solution was prepared in a 0.15 *M* potassium iodide solution. To this, 10 mc. of I^{131} was added as sodium iodide. The solution was incubated at 37° to allow equilibration between the I_2 and the I^{131} ions. The latter solution was usually made up simultaneously with the former one. On the following day, 40 ml. of the fibrinogen solution was mixed in an ice bath with 40 ml. borate buffer of pH 8.58 (equal volumes of 0.5 *M* boric acid and 0.125 *M* sodium tetraborate) and 100 ml. 40% urea solution. To this mixture 20 ml. of the precooled iodine solution was added. After thirty minutes, the remaining free iodine was reduced with a 10% sodium thiosulfate solution. Urea, iodide ions, and sodium thiosulfate were then removed by overnight dialysis against double distilled water in the cold. Iodinated fibrinogen was then precipitated by 5 ml. of 0.5 *M* acetate buffer (equal volumes of 0.5 *M* acetic acid and 0.5 *M* sodium acetate) of pH 4.6. The precipitate was redissolved in 10 ml. of distilled water and adjusted to pH 7.5 with 0.1 *N* sodium hydroxide. Fresh I^{131}-labeled fibrinogen was prepared for each experimental series.

In all animals, prothrombin times were determined periodically from oxalated plasma with the aid of Simplastin (5). Clotting index was calculated as the ratio of the value before infusion of the compounds under test and the experimental values. This same determination was repeated after adding 0.1 ml. of a 2.5% fibrinogen solution to the test mixture. This procedure was used to get an indication as to the role of decrease in fibrinogen level in changes of the clotting index. Fibrinogen values were determined by the method of Shea (6).

Blood pressure and EKG's were taken in all animals.

The following enzymes were tested in this study: pancreatic protease, ficin, carboxy peptidase, and papain. Crude lyophilized pancreatic protease was obtained from the Worthington Laboratories. Crystalline ficin was supplied by the Mann Research

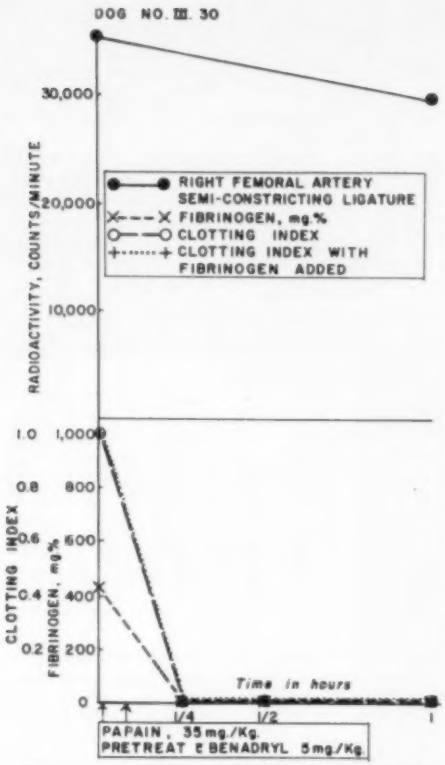

Fig. 5.—Effect of papain, 35 mg./Kg., on clotting index, fibrinogen level, and I^{131}-labeled clot in dog pretreated with Benadryl, 5 mg./Kg.

Laboratories. Carboxypeptidase (triple crystallized) was prepared according to the method of Anson (7) by the Worthington Laboratories. Papain was obtained from Merck and Co. All of these compounds were tested for *in vitro* fibrinolytic activity by the method of Loomis, George, and Ryder (8). The results of these *in vitro* tests are summarized in Table II. The doses given to the animals were chosen to be the maximal quantities which do not cause death within at least four hours. The time of infusion was selected to be the shortest period which does not cause irreversible fall in blood pressure.

RESULTS

Figure 1 shows the results of a control experiment. It can be seen that only slight spontaneous fibrinolysis occurs in ninety-six hours. Figure 2 shows the results of an experiment in which 25 mg./Kg. of pancreatic protease has been administered. There is a sharp fall of fibrinogen level and clotting index, even in the presence of added fibrinogen. Although in many experiments the blood became incoagulable, no significant fibrinolysis occurred. Table ID[1] indicates the abbreviations used in all tables. Table IID[1] presents the data of the experiments with pancreatic protease and the corresponding control studies. Figure 3 shows the results of an experiment with ficin. Again, there can be seen a sharp fall in clotting index and fibrinogen values, but no evidence is present of fibrinolysis. Table IVD[1] contains the detailed data of the ficin experiments together with controls. Figure 4 graphs the results of an experiment with carboxypeptidase. No significant change is observed in clotting index or fibrinogen level, nor is there more fibrinolysis than in the control animals. The detailed data are tabulated in Table VD[1]. This same table contains results obtained with papain. A typical papain experiment is shown in Fig. 5. Again, there is a precipitous fall in fibrinogen level and clotting index with and without fibrinogen, but there is no evidence for fibrinolysis.

Table I groups all the above results according to the percentage of clots exhibiting the indicated percentage lysis values at various time intervals. Table II summarizes all experiments by indicating the mean percentage lysis at various time intervals obtained with the compounds tested. This table also contains the results of *in vitro* fibrinolytic assays. It can be seen that none of the enzymes tested had any important *in vivo* fibrinolytic activity. The slight increase over control values shows relation to the *in vitro* activity.

Except for carboxypeptidase, all of the compounds tested produced fall in blood pressure. This could not be prevented by pretreatment with 5 mg./Kg. Benadryl. The EKG changes were mostly insignificant; occasionally T wave reversion was seen with protease.

DISCUSSION AND SUMMARY

Four proteolytic enzymes, crude pancreatic protease, ficin, carboxypeptidase, and papain, have been tested for *in vivo* fibrinolytic activity upon intravenous administration in dogs. A quantitative method was used for evaluation based on the production of clots with I^{131}-labeled fibrinogen and continuous recording of radioactivity over the thrombosed blood vessel segment. None of the enzymes tested produced significant lysis. All except carboxypeptidase decreased clotting index and fibrinogen level. This often resulted in complete incoagulability of the blood.

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A Note on the Volatile Oil from the Fruits of *Lomatium Grayi**

By FRANK A. PETTINATO†, LOUIS FISCHER, and NATHAN A. HALL

MANY SPECIES of the genus *Lomatium* grow prolifically in some areas of the state of Washington and have been the subject of only a limited number of studies. A literature review revealed that *Lomatium Grayi* was one species which had not been previously investigated. As part of a study of this genus in an effort to find constituents of medicinal or economic value, a preliminary investigation of the volatile oil from the fruits of *Lomatium Grayi* Coult. and Rose was undertaken.

The fruits of the plant were collected in June 1956 near Klickitat, Washington, dried in air, and ground in a Wiley mill through a 1-mm. screen immediately before use. Two hundred-thirty milliliters of oil was steam distilled from 7.1 Kg. of the ground fruit for a yield of 3% on an air-dried basis. The pale yellow oil was dried over anhydrous sodium sulfate and the physical constants and the results of the chemical analysis shown in Table I were obtained.

TABLE I.—PHYSICAL AND CHEMICAL PROPERTIES OF *L. Grayi* OIL

Specific gravity, 25°/25°	0.8782
Optical rotation, 25°	−16.05
Refractive index, 23°	1.4848
Acid number	0.83
Ester number	24.5
Ester number (after acetylation)	46.9
Esters (as bornyl acetate)	8.6%
Alcohols (as borneol)	13.4%
Aldehydes and ketones (by hydroxylamine method, assuming average M. W. of 150)	4.0%

TABLE II.—PHYSICAL PROPERTIES OF *L. Grayi* OIL FRACTIONS

Fraction	Boiling Range, °C.	Distillate, %	$(n)_D^{29}$	d_{25}^{25}	$[\alpha]_D^{25}$
1	To 46	1	1.4659	...	−19.30
2	46–53	1	1.4742	...	−10.60
3	53–55	2	1.4822	0.848	− 7.00
4	55–57	24	1.4822	0.852	− 4.90
5	57–58	24	1.4820	0.849	− 4.00
6	58	16	1.4805	0.848	− 3.10
7	58–60	11	1.4782	0.848	− 1.80
8	60–75	1	1.4772	...	− 2.80
Residue and loss	...	20	...	...	...

Seventy-five milliliters of the oil was fractionally distilled from a Todd Fractionation Assembly, Model A equipped with a 0.5 x 90 cm. column (with monel spiral) at 10 mm. pressure and a 10:1 reflux ratio. The fractions and their physical characteristics are shown in Table II.

The further characterization and identification of the constituents of the oil are in progress.

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† Present address: School of Pharmacy, Montana State University, Missoula.

The authors wish to thank Dr. A. E. Kruckeberg, Dept. of Botany, University of Washington, Seattle, and Dr. Lincoln Constance, Dept. of Botany, University of California, Berkeley, for the botanical identification.

Communication to the Editor

Gastric Antacids

Sir:

Subsequent to the publication of the article, "The Antisecretory and Antipeptic Activity of Gastric Antacids in the Histamine-Stimulated Rabbit" by Packman, *et al.* [THIS JOURNAL, **48**, 46(1959)], we noticed several errors which should be corrected. They are as follows:

$$\text{VPN Index} = \frac{\%\text{ Reduction in gastric secretion} \times \left[1 + \frac{\%\text{ Reduction in proteolytic activity}}{100} + \frac{\%\text{ Animals maintained in pH range 3–5.5}}{100}\right]}{\text{Acid consuming power}}$$

Immediately following the VPN indexes should be: DASC, 8.9; calcium carbonate, 4.0; sodium bicarbonate, 4.1; and aluminum hydroxide, 0.1.

We regret that these errors were not corrected in galley proof. We wish to assure you that these errors resulted from our oversight and not yours or that of the proofreader.

E. W. PACKMAN,
D. D. ABBOTT,
B. TRABIN,
and J. W. E. HARRISSON
La Wall and Harrisson Research Laboratories,
Philadelphia, Pa.

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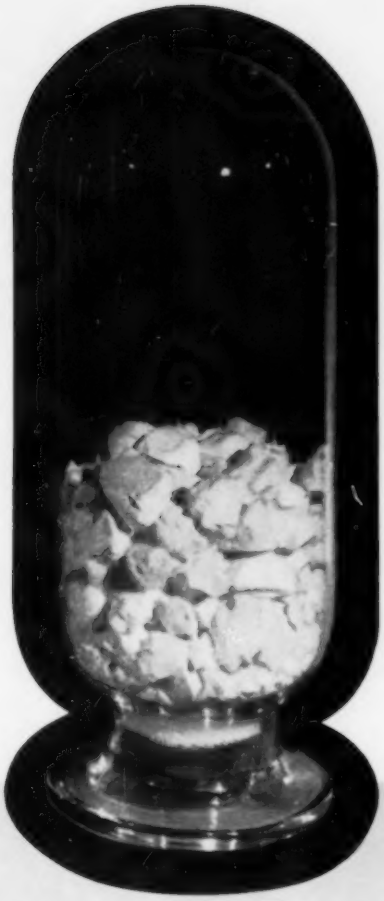

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